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<b>(54) Title:</b> IDENTIFICATION AND CHARACTERIZATION OF INTERACTING MOLECULES  <b>(57) Abstract</b>  The present invention relates to an improved method for the identification and optionally the characterization of interacting molecules designed to detect positive clones from the rather large numbers of false positive clones isolated by two-hybrid systems. The method of the invention relies on a novel combination of selection steps used to detect clones that express interacting molecules from false positive clones. The present invention further relates to a kit useful for carrying out the method of the invention. The present invention provides for parallel, high-throughput or automated interaction screens for the reliable identification of interacting molecules.		

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## IDENTIFICATION AND CHARACTERIZATION OF INTERACTING MOLECULES

The present invention relates to an improved method for the identification and optionally the characterization of interacting molecules designed to detect positive clones from the rather large numbers of false positive clones isolated by two-hybrid systems. The method of the invention relies on a novel combination of selection steps used to detect clones that express interacting molecules from false positive clones. The present invention further relates to a kit useful for carrying out the method of the invention. The present invention provides for parallel, high-throughput or automated interaction screens for the reliable identification of interacting molecules.

Protein-protein interactions are essential for nearly all biological processes like replication, transcription, secretion, signal transduction and metabolism. Classical methods for identifying such interactions like co-immunoprecipitation or cross-linking are not available for all proteins or may not be sufficiently sensitive. Said methods further have the disadvantage that only by a great deal of energy, potentially interacting partners and corresponding nucleic acid fragments or sequences may be identified. Usually, this is effected by protein sequencing or production of antibodies, followed by the screening of an expression-library.

An important development for the convenient identification of protein-protein interactions was the yeast two-hybrid (2H) system presented by Fields and Song (1989). This genetic procedure not only allows the rapid demonstration of in vivo interactions, but also the simple isolation of corresponding nucleic acid sequences encoding for the interacting partners.

The yeast two-hybrid system makes use of the features of a wide variety of eukaryotic transcription factors which carry two separable functional domains: one DNA binding domain as well as a second domain which activates the RNA-polymerase complex (activation domain). In the classical 2H system a so-called "bait" protein comprising of a DNA binding domain (GAL4bd or lex A) and a protein of interest "X" are expressed as a fusion protein in yeast. The same yeast cell also simultaneously expresses a so called "fish" protein comprising of an activation domain (GAL4ad or VP16) and a protein "Y". Upon the interaction of a bait protein with a fish protein, the DNA binding and activation domains of the fusion proteins are brought into close proximity and the resulting protein complex triggers the expression of the reporter genes, for example, HIS3 or lacZ. Said expression can be easily monitored by cultivation of the yeast cells on selective medium without histidine as well as upon the activation of the lacZ gene. The genetic sequence encoding, for example, an unknown fish protein, may easily be identified by isolating the corresponding plasmid and subsequent sequence analysis. Meanwhile, a number of variants of the 2H system have been developed. The most important of those are the "one hybrid" system for the identification of promoter binding proteins and the "tri-hybrid" system for the identification of RNA-protein-interactions (Li and Herskowitz, 1993; SenGupta et al., 1996; Putz et al., 1996). It is understood in the art that to identify, detect or assay the variety of interactions found in biological systems, different 2H systems must be employed. Indeed, other 2H technologies have been developed to enable protein-protein interactions to be investigated in other organisms and/or different cell compartments. For example, in mammalian cells (Rossi et al, 1997; PNAS 94:8405-8410), in bacterial cells (Karimova et al., 1998; PNAS 95:5752-5756), in the cytoplasm of yeast cells (Johnsson & Varshavsky; 1996 US5503977) and in the periplasmic space of yeast cells (Fowlkes et al., 1998; US 5789184).



These 2H systems for the identification of protein-protein-interaction, have, until today, only been carried out on a laboratory scale. The various steps of these systems need to be conducted serially. They are, therefore, quite time consuming. As a consequence, these 2H systems have so far proven unsuitable for the analysis of eukaryotic library vs library screens to investigate protein-protein networks. Although recent developments have taken into account these disadvantages (Bartel et al., 1996), a successful large scale search of interacting proteins, for example on the basis of a eukaryotic library vs. library screen, has not been reported. More importantly also, 2H systems suffer from the serious drawback that many false-positive clones not representing any interactions between binding partners are isolated. This is particularly inconvenient in cases where large numbers of clones are to be analyzed because in the case of a eukaryotic library vs library screen it is typical that several hundreds of thousands of clones have to be analyzed for the investigation of protein-protein networks.

The technical problem underlying the present invention was therefore to overcome these prior art difficulties and to furnish a system that reliably produces clones that express interacting molecules. This system should, moreover, be suitable for large-scale library vs library screens using a parallel, high-throughput or automated approach.

The solution to said technical problem is achieved by providing the embodiments characterized in the claims.

Accordingly, the present invention relates to a method for the identification of at least one member of a pair or complex of interacting molecules, comprising:

- (a) providing host cells containing at least two genetic elements with different selectable and counterselectable markers, said genetic elements each comprising genetic information specifying one of said members, said host

- cells further carrying a readout system that is activated upon the interaction of said molecules;
- (b) allowing at least one interaction, if any, to occur;
  - (c) selecting for said interaction by transferring progeny of said host cells to
    - (ca) at least two different selective media, wherein each of said selective media allows growth of said host cells only in the absence of at least one of said counterselectable markers and in the presence of a selectable marker; and
    - (cb) a further selective medium that allows identification of said host cells only on the activation of said readout system ;
  - (d) identifying host cells containing interacting molecules that
    - (da) do not activate said readout system on any of said selective media specified in (ca); and
    - (db) activate the readout system on said selective medium specified in (cb); and
  - (e) identifying at least one member of said pair or complex of interacting molecules.

Preferably, said interaction is a specific interaction.

The terms "identification" and "identifying", as used in accordance with the present invention, relate to the ability of the person skilled in the art to detect positive clones that express interacting molecules from false positive clones due to the activation of the readout system on the selective media and optionally additionally to characterize at least one of said interacting molecules by one or a set of unambiguous features. Preferably, said molecules are characterized by the DNA sequence encoding them, upon nucleic acid hybridization or isolation and sequencing of the respective DNA molecules. Alternatively and less preferred, said molecules may be characterized by different features such as molecular weight, isoelectric point and, in the case of proteins, the N-terminal

amino acid sequence etc. Methods for determining such parameters are well known in the art.

Preferably, said members specified by said genetic elements are connected to a further entity that will upon the interaction activate or contribute to the activation of said read out system. It is further preferred that said entity is conserved for each type of genetic element and that different types of genetic elements comprise different entities. It is additionally preferred that said member of said pair or complex of interacting molecules forms, when transcribed as RNA from said genetic element, an RNA transcript fused with RNA specifying said entity. Most preferably, said fused RNA transcript is translated to form a fusion protein comprising said member fused to said entity. As will be elaborated further herein below, said entity may be in one type of genetic element a DNA sequence encoding a DNA-binding domain and in a different type of genetic element a transactivating protein domain. Preferably, said genetic elements are vectors such as plasmids. Alternatively, interaction between two fusion proteins may result in a functional entity with reconstituted enzymatic activity, for example the bacterial chloramphenicol acetyltransferase protein (CAT) (Seed & Sheen, 1988 Gene 67:271-277). The at least two genetic elements comprised in said host cell are preferentially vectors from a library such as a cDNA or genomic library. Thus, the method of the invention allows the screening of a variety of host cells wherein the vector portion of said genetic elements is preferably the same for each type of genetic element whereas the potentially interacting molecules are representatives of a library and, thus, as a rule and in case that the library has not been amplified, may differ in each host cell. In this connection the term "type of genetic element" refers to an element characterized by comprising the same entity, selectable and counterselectable markers.

Preferably, the "interaction" of said molecules is specific and characterized by a high binding constant. However, the term "interaction" may also refer to a binding between molecules with a lower binding constant which, however, must be sufficient to activate the readout system. The interaction that is detectable by the method of the invention preferably leads to the formation of a functional entity having a biological, physical or chemical activity which was not present in said host cell before said interaction occurred.

Said interaction may lead to the formation of a functional transcriptional activator comprising a DNA-binding and a transactivating protein domain and which is capable of activating a responsive moiety that drives the activation of said readout system. For example, said moiety may be a promoter.

Alternatively, said interaction may lead to a detectable fluorescence resonance energy transfer obtained by the interaction of fusion proteins containing, for example, the GFP type a and GFP type b fluorescent proteins (Cubbitt et al., 1995; Heim & Tsien, Curr Biol. 1996 6:178-182). Said interaction may also alternatively lead to the reconstitution of a functional enzyme, for example  $\beta$ -galactosidase (Rossi et al., 1997) or adenylate cyclase (Karimova et al., 1998). These embodiments will be preferred for the study of interactions in host-cell types other than yeast.

In a further embodiment, said interaction may lead to a detectable modification of a substrate by an enzyme such as a color reaction obtained by the cleavage of a propeptide by an enzyme. In all these embodiments of the invention, it is understood that the interacting molecules are preferably directly fused to the molecules driving the readout system.

The term "growth" on selective media "in the absence of at least one of said counter-selectable markers" refers to the fact that a population of host cells containing at least one

of genetic elements is placed on said selective media but only those progeny of the host cells in the overall population that have lost the relevant genetic element are able to grow. For example, when a yeast strain which is resistant to the drug canavanine ( $\text{can}^r$ ) and which also contains a plasmid carrying the wild-type CAN1 gene (Hoffmann, 1985) is placed on a selective medium containing canavanine, only those progeny of the yeast strain that have lost the plasmid carrying the CAN1 gene are able to grow, because this gene confers sensitivity to canavanine in yeast cells.

With reference to step (ca), it should be noted that each of the at least two selective media would comprise at least one counterselectable compound such as cycloheximide wherein the counterselectable compound would be different in the different selective media; they would further typically lack a compound complementing for an auxotrophic marker or comprise an antibiotic. The compound or antibiotic may be the same for the various selective media. Preferably, at least one is different.

The method of the present invention provides a highly effective tool for selecting against false positive clones that have proven to dramatically reduce the overall usefulness of the two-hybrid system. For example, by inclusion of a marker counterselecting for the absence of a genetic element that specifies one of a pair of the potentially interacting partners, clones that will grow and therefore only carry the second genetic element specifying the second partner can now be tested for the activation of the readout system. If the clone containing only the fusion protein encoded by the second genetic element activates the readout system in the absence of the other genetic element, then it will be classified as a false positive. By counterselecting for the absence of the second genetic element, the same test is applied to the first genetic element. Thus, only clones that activate the readout system in the presence of both or all genetic elements, but do

not activate the read out system when either of the genetic elements is lost are classified as positives.

The advantages associated with the method of the invention have a significant impact in particular on the number of clones that express potentially interacting partners that can conveniently be analyzed. For example, even work on the laboratory scale will be more effective since positive clones that express interacting partners can be easily and unambiguously discriminated from false positive clones without the generation of additional strains. In contrast, to detect false positive clones using the state of the art yeast two-hybrid system, plasmids that encode fish proteins usually need to be isolated and retransformed into yeast cells harboring plasmids that encode unrelated bait proteins. Further, the enormous number of false positive clones that would be isolated when using the classical two-hybrid system on a large scale, yet are discriminated by the method of this invention no longer precludes an effective high through-put analysis of clones. In the long run, it is expected that the method of the present invention is especially advantageous for a high throughput analysis of a large number of yeast clones containing interacting molecules since many specific interactions and the individual members of these interactions can be identified in a parallel and automated approach.

Some investigators have noted the problem of identifying false positive clones when applying the yeast two-hybrid system in the past. Bartel et al. (1996) described a method for the elimination of false positives by replica plating clones that express one fusion protein from SD-leu and SD-trp plates, to SD-his plates. Clones that showed growth on the SD-his plates were identified as false positives and were subsequently not used for interaction mating. The disadvantage of this method is that the procedure is labor intensive because yeast strains expressing the fish proteins, the bait proteins and the potentially interacting fish and bait proteins all must be

generated and analyzed. The use of the counterselectable system described in this invention has the advantage that only one strain which expresses the potentially interacting fusion proteins is generated and must be analyzed.

Other strategies have been proposed to eliminate false positive clones from 2H systems (Vidal et al., 1996a; Nandabalan et al., 1997). However, these systems all require that the readout system that is assayed for activity comprises at least one reporter gene that is transcribed on reconstitution of DNA binding and transactivating fusion proteins. Indeed, although mostly claiming to be applicable to all types of cells, these systems have been designed towards the specific biological properties of the yeast two-hybrid system. The method of invention described herein is not limited to eliminating false positive clones expressing single DNA binding or activation domain fusion proteins that can activate the reporter system. On the contrary, it can be used to eliminate false positive clones in 2H systems other than yeast two-hybrid, which is of advantage when interaction screens are conducted in for example, other host-cell types.

A schematic overview of one embodiment of the method of the invention is provided in Figure 6. For the parallel analysis of a network of protein-protein interactions with the method of the invention, a library of plasmid constructs that express DNA binding domain and activation domain fusion proteins is provided. These libraries may consist of specific DNA fragments or a multitude of unknown DNA fragments ligated into the improved binding domain and activating domain plasmids of the invention containing different selectable and counterselectable markers. Both libraries are combined within yeast cells by transformation or interaction mating, and yeast strains that express potentially interacting proteins are selected on selective medium lacking histidine. The selective markers TRP1 and LEU2 maintain the plasmids in yeast strains grown on selective media, whereas CAN1 and CYH2 specify the

counter-selectable markers that select for the loss of each plasmid. HIS3 and lacZ represent selectable markers integrated into the yeast genome, which are expressed on activation by interacting fusion proteins.

The readout system is, in the present case, both growth on medium lacking histidine and enzymatic activity of  $\beta$ -galactosidase which can be subsequently screened. It is to be understood, however, that the readout system may rely on only one marker such as HIS3. Yet, the combination of two components that constitute the readout system in many cases allows a more ready interpretation of results, in particular if one of the components, when activated, effects a change in color. A colony picking robot is used to pick the resulting yeast colonies into individual wells of 384-well microtiter plates containing selective medium lacking histidine, and the resulting plates are incubated at 30°C to allow cell growth. The interaction library contained in microtiter plates can be optionally replicated and stored. The resulting interaction library is investigated to detect positive clones that express interacting proteins and discriminate them from false positive clones using the method of the invention. Using a spotting robot, cells are transferred to replica membranes which are subsequently placed onto one each of the selective media SD-leu-trp-his, SD-leu+CAN and SD-trp+CHX. After incubation on the selective plates, the clones grown on the membranes are subjected to a  $\beta$ -Gal assay and a digital image from each membrane is obtained with a CCD camera which is then stored on computer. Using digital image processing and analysis clones that express interacting fusion proteins can be identified by considering the pattern of  $\beta$ -Gal activity from clones grown on the various selective media. The individual members comprising interactions can then be identified by one or more techniques, including PCR, sequencing, hybridization, oligofingerprinting or antibody reactions. An actual experiment carried out along the schematic route presented in Figure 6 is shown in Figure 5 to Figure 22.



The genetic elements specified here and above may further and advantageously be equipped with at least two different selection markers functional in bacteria such as E.coli. Such selection markers, for example aphA (Pansegrau et al., 1987) or bla allow the easy separation of said genetic elements upon retransformation into E.coli strains.

In a preferred embodiment of the method of the present invention said pair or complex of interacting molecules is selected from the group consisting of RNA-RNA, RNA-DNA, RNA-protein, DNA-DNA, DNA-protein, protein-protein, protein-peptide, or peptide-peptide interactions.

Accordingly, the method of the invention is applicable in a wide range of biological interactions. For example, the invention will be useful in identifying peptide-protein or peptide-peptide-interactions by employing synthetic peptide libraries (Yang et al., 1995).

Two applications of interests are the application of a large scale two-hybrid system for the detection of protein-protein interactions involved in medically relevant pathways which may be useful as diagnostic or therapeutic targets for the treatment of disease, and a large scale tri-hybrid system which is one example of said complex of interacting molecules mentioned herein above for the identification of, for example, novel post-transcriptional regulators and their binding sites (SenGupta et al., 1996; Putz et al., 1996). In this regard it should be noted that a complex, in accordance with the invention may comprise more than three interacting molecules. Furthermore, such a complex may be composed of biologically or chemically different members. For example, to identify interacting RNA binding proteins and RNA molecules, a plasmid expressing a LexA-HIV-1Rev protein, a plasmid transcribing an RNA sequence in fusion with the Rev responsive element and a plasmid expressing a potentially RNA-interacting protein in

fusion with an activation domain may be present in one cell. The plasmids encoding the RNA fusion molecule and the activation domain fusion protein must contain different selectable and counterselectable markers according to the method of the invention. If the RNA fusion molecule interacts with the respective two fusion proteins, the readout system is activated. To test whether the RNA fusion molecule or the activation domain fusion protein interact, the method of the invention is used to investigate the activation of the readout system in the absence of either of these fusion molecules.

In a further preferred embodiment, said genetic elements are plasmids, artificial chromosomes, viruses or other extrachromosomal elements.

Whereas it is preferred, due to the easy handling, to employ plasmids that specify the genetic elements in accordance with the present invention, the persons skilled in the art will be able to devise other systems that carry said genetic elements and that are identified above.

In an additional preferred embodiment, said readout system is a detectable protein. A number of readout systems are known in the art and may, if necessary, be adapted to be useful in the method of the invention.

Most preferably, said detectable protein is that encoded by the gene lacZ, HIS3, URA3, LYS2, sacB or HPRT, respectively. As is well known in the art, the expression of the  $\beta$ -gal enzyme in yeast can be used for the formation of a detectable blue colony after incubation in X-Gal solution. Of course, the method of the invention is not restricted for use of only one readout system. On the contrary, if desired, a number of such readout systems may be combined. Said combination of a number of readout systems is, in accordance with the present invention, also comprised by the term "readout system". Such a

combination will provide an additional safe guard for the identification of clones containing interacting partners.

Although the two-hybrid system has been developed in yeast, the method of the invention can be carried out in a variety of host systems. Preferred of those are yeast cells, bacterial cells (Karimova et al., 1998), mammalian cells (Wu et al. 1996, Rossi et al., 1997), insect cells or plant cells. Preferably, the bacterial cells are E. coli cells. Of course, the genetic elements may be engineered and prepared in one host organism and then, e.g. by employing shuttle vectors, be transferred to a different host organism where it is employed in the method of the invention.

In another preferred embodiment, the method of the present invention comprises transforming or transfecting said host cell with at least one of said genetic elements prior to step (a).

Whereas the person skilled in the art may initiate the identification method of the invention starting from fully transformed or transfected host cells, he may wish to first generate such host cells in accordance with the aim of his research or commercial interest. For example, he may wish to generate a certain type of library first that he intends to screen against a second library already present in said host cells. Alternatively, he may have in mind to generate two or more different libraries that he wants to screen against each other. In this case, he would need to first transform said host cells, simultaneously or successively, with both or all types of genetic elements.

In another preferred embodiment, said host cells with said genetic elements are generated by cell fusion, conjugation or interaction mating.

The biological principal of counter-selection referred to above is well known in the art. Accordingly, the person skilled in the art may chose from a variety of such counter-selectable markers. Preferably, said markers are CAN1, CYH2, LYS2, URA3, HRPT or sacB.

It is further preferred in accordance with the present invention that said selectable markers are auxotrophic or antibiotic markers.

It is important to note that some of the markers that are used as a readout system, may also be used as selectable markers. It is further important to note that one and the same marker can not be used as selectable marker and as part of the readout system at the same time.

Most preferably, said auxotrophic or antibiotic markers are selected from LEU2, TRP1, URA3, HIS3, ADE2, LYS2 and Zeocin.

Planning of experiments may require that the test for interaction need not be done immediately after the provision of host cells and, possibly, the occurrence of the interactions. In such cases, the researcher may wish to store the transformed host cells for further use. Accordingly, a further preferred embodiment of the invention relates to a method wherein progeny of host cells obtained in step (b) are transferred to a storage compartment.

In particular in cases where a large number of clones is to be analyzed, said transfer is advantageously effected or assisted by automation or a picking robot. Naturally, other automation or robot systems that reliably pick progeny of said host cells into predetermined arrays in the storage compartments may also be employed.

The host cells will, in this embodiment, be propagated in said storage compartment and provide further progeny for the

additional tests. Preferably, replicas of said storage compartment maintaining the array of clones are set up. Said storage compartments comprising the transformed host cells and the appropriate media may be maintained in accordance with conventional cultivation protocols. Alternatively, said storage compartments may comprise an anti-freeze agent and therefore be appropriate for storage in a deep-freezer. This embodiment is particularly useful when the evaluation of potential interacting partners is to be postponed. As is well known in the art, frozen host cells may easily be recovered upon thawing and further tested in accordance with the invention. Most preferably, said anti-freeze agent is glycerol which is preferably present in said media in an amount of 3 - 25% (vol/vol).

In a further particularly preferred embodiment of the method of the invention, said storage compartment is a microtiter plate. Most preferably, said microtiter plate comprises 384 wells. Microtiter plates have the particular advantage of providing a pre-fixed array that allows the easy replicating of clones and furthermore the unambiguous identification and assignment of clones throughout the various steps of the experiment. The 384 well microtiter plate is, due to its comparatively small size and large number of compartments, particularly suitable for experiments where large numbers of clones need to be screened.

Depending on the design of the experiment, the host cells may be grown in the storage compartment such as the above microtiter plate to logarithmic or stationary phase. Growth conditions may be established by the person skilled in the art according to conventional procedures. Cell growth is usually performed between 15 and 45 degrees Celsius.

Transfer of said host cells in step (c) is made or assisted by automation, by using a spotting robot or by using a pipetting or micropipetting device. How such a spotting robot may be

devised and equipped is, for example, described in Lehrach et al. (1997). Naturally, other automation or robotic systems that reliably create ordered arrays of clones may also be employed.

Most preferably, said transfer is made to a planar carrier which is subsequently placed on the at least three selective media as specified in steps (ca) and (cb). Alternatively, said transfer of said host cells may be made to the planar carrier already placed on the selective media or said transfer may be made directly to the selective media.

Most advantageously, said transfer is effected in a regular grid pattern at densities of 1 to 1000 clones per square centimeter. The progeny of said host cells may be transferred to a variety of planar carriers. Most preferred is a membrane which may, for example, be manufactured from nylon, nitro-cellulose or PVDF.

The selective media used for growth of appropriate clones may be in liquid or in solid form. Preferably, said selective media when used in conjunction with a spotting robot and membranes as planar carriers are solidified with agar on which said spotted membranes are subsequently placed. Alternatively, and also preferably, said selective media when in liquid form are held within microtiter plates and said transfer is made by replication.

Referring now to the step (d) of the method of the invention, the readout system can be analyzed by a variety of means. For example, it can be analyzed by visual inspection, radioactive, chemiluminescent, fluorescent, photometric, spectrometric, infra red, colourimetric or resonant detection.

Preferably, said identification of host cells that express interacting fusion proteins is effected by visual means from consideration of the activation state of said readout system

of clones grown on the at least three selective media as specified in steps (ca) and (cb).

Also preferably, said identification of host cells that express interacting fusion proteins in step (d) is effected or assisted by digital image storage, analysis or processing. In this embodiment, positive clones which are preferably arrayed on a planar carrier such as a membrane are identified by comparison of digital images obtained from the membrane after activation of said readout system on said selective media specified in (ca) and (cb).

Most preferably, the identity of positive host cells and false positive host cells are stored on computer, for example within a relational database.

Identification of the at least one member of the pair or complex of interacting molecules may be effected by a variety of means. For example, molecules can be characterized by nucleic acid hybridization, oligonucleotide hybridization, nucleic acid or protein sequencing, restriction digestion, spectrometry or antibody reaction. Once the first member of an interaction has been identified, the second member or further members can also be identified by any of the above methods. Preferably the identification of at least one member of an interaction is effected by nucleic acid hybridization, antibody binding or nucleic acid sequencing.

If nucleic acid hybridization is to be carried out, the nucleic acid molecules comprised in the host cell and encoding for at least one of the interacting molecules is preferably affixed to a planar carrier. As is well known in the art, said planar carrier to which said nucleic acid may be affixed, can be for example, a Nylon-, nitrocellulose- or PVDF membrane, glass or silica substrates (DeRisi et al. 1996; Lockhart et al. 1996). Said host cells containing said nucleic acid may be transferred to said planar carrier and subsequently lysed on

the carrier and the nucleic acid released by said lysis is affixed to the same position by appropriate treatment. Alternatively, progeny of the host cells may be lysed in a storage compartment and the crude or purified nucleic acid obtained is then transferred and subsequently affixed to said planar carrier. Advantageously, said nucleic acids are amplified by PCR prior to transfer to the planar carrier. Most preferably said nucleic acid is affixed in a regular grid pattern in parallel with additional nucleic acids representing different genetic elements encoding interacting molecules. As is well known in the art, such regular grid patterns may be at densities of between 1 and 50 000 elements per square centimeter and can be made by a variety of methods. Preferably, said regular patterns are constructed using automation or a spotting robot such as described in Lehrach et al. (1997) and Maier et al. (1997) and furnished with defined spotting patterns, barcode reading and data recording abilities. Thus it is possible to correctly and unambiguously return to stored host cells containing said nucleic acid from a given spotted position on the planar carrier. Also preferably, said regular grid patterns may be made by pipetting systems, or by microarraying technologies as described by Shalon et al. (1996), Schober et al (1993) or Lockart et al. (1996). Identification is, again, advantageously effected by nucleic acid hybridization.

Using a detectable nucleic acid probe of interest, homologous nucleic acids which are affixed on the planar carrier can be identified by hybridization. From the spotted position of said homologous identified nucleic acid on the planar carrier, the corresponding host cell in the storage compartment can be identified which contains both or all members of the interaction. The for example second member of the interaction can now be identified by any of the above methods. For example, by use of a radioactively labeled Ras probe, homologous nucleic acids on the planar carrier can be identified by hybridization. The Ras interacting proteins can



now be identified from the corresponding host cell that contains both the first genetic element homologous to the Ras probe and the second genetic element encoding for these Ras interacting proteins.

If multiple oligonucleotide hybridizations are carried out on the nucleic acids affixed to the planar carrier, oligofingerprints of all genetic elements encoding the interacting proteins can be obtained. These oligofingerprints can be used to identify all members of the interactions or those members that belong to specific gene families, as described in Maier et al. (1997).

Advantageously, the nucleic acid molecules that encode the interacting proteins are, prior to identification such as by DNA sequencing, amplified by PCR or in said genetic elements in host cells and preferable in *E. coli*. Amplification of said genetic elements is conducted by multiplication of the *E. coli* cells and isolation of said genetic elements. Methods of identifying the nucleic acids that encode interacting proteins by DNA sequencing and analysis are well known in the art. By amplifying and sequencing the nucleic acids that encode for both or all members of an interaction from the same clone, the identity of both or all members of the interaction can be determined.

If a specific antibody is to be used to determine whether a protein of interest is expressed as a fusion protein within an interaction library, it is advantageous to affix all fusion proteins expressed from the interaction library on to a planar carrier. For example, clones of the interaction library that express fusion proteins can be transferred to a planar carrier using a spotting robot as described in Lehrach et al (1997). The clones are subsequently lysed on the carrier and released proteins are affixed onto the same position. Using, for example, an anti-HIP1-antibody (Wanker et al. 1997), clones from the interaction library that contain HIP1 fusion proteins

and an unknown interacting fusion protein can be identified. The unknown member of the interacting pair of molecules can now be identified from the corresponding host cell by any of the above methods. The antibodies used as probes may be directly detectably labeled. Alternatively, said antibodies may be detected by a secondary probe or antibody which may be specific for the primary antibody. Various alternative embodiments using, for example, tertiary antibodies may be devised by the person skilled in the art on the basis of his common knowledge.

Most advantageously, when said identification of members comprising an interaction is effected using said regular grids, a digital image of the planar carrier after hybridization or antibody reaction is obtained and analysis is effected by digital image storage, processing or analysis using an automated or semi-automated image analysis system, such as described in Lehrach et al. (1997).

Most preferably, the information comprising the identity of the host cell and the identity of the interacting molecules expressed by the genetic elements contained within the host cell are stored on a computer, for example within a relational database.

In accordance with the present invention, it is additionally preferred prior to step (a) that a preselection against clones that express a single molecule able to activate the readout system is carried out on culture media comprising a counterselective compound, for example 5-fluoro orotic acid, canavanine, cycloheximide or  $\alpha$ -amino-adipate.

In this embodiment, for example, the URA3 gene is incorporated as a component of the readout system. Clones containing only one of said genetic elements are placed on a selective medium comprising 5-fluoro orotic acid (5-FOA). In the case that

clones that express a single molecule able to activate the readout system, 5-FOA is converted into the toxic 5-fluorouracil. Accordingly, host cells containing auto-activating molecules will die on the selective medium containing 5-FOA.

It is further important to note that the marker used for said preselection cannot be used as a selectable or counterselectable marker at the same time.

The present invention also relates to a method for the production of a pharmaceutical composition comprising formulation said at least one member of the interacting molecules identified by the method of the invention in a pharmaceutically acceptable form.

Said pharmaceutical composition comprises at least one of the aforementioned compounds identified by the method of the invention, either alone or in combination, and optionally a pharmaceutically acceptable carrier or excipient. Examples of suitable pharmaceutical carriers are well known in the art and include phosphate buffered saline solutions, water, emulsions, such as oil/water emulsions, various types of wetting agents, sterile solutions etc. Compositions comprising such carriers can be formulated by conventional methods. These pharmaceutical compositions can be administered to subject in need thereof at a suitable dose. Administration of the suitable compositions may be effected by different ways, e.g., by intravenous, intraperitoneal, subcutaneous, intramuscular, topical or intradermal administration. The dosage regimen will be determined by the attending physician and other clinical factors. As is well known in the medical arts, dosages for any one patient depends upon many factors, including the patient's size, body surface area, age, the particular compound to be administered, sex, time and route of administration, general health, and other drugs being administered concurrently. Dosages will vary but a preferred dosage for intravenous

administration of DNA is from approximately  $10^6$  to  $10^{22}$  copies of the nucleic acid molecule. Proteins or peptides may be administered in the range of 0,1ng to 10mg per kg of body weight. The compositions of the invention may be administered locally or systematically. Administration will generally be parenterally, e.g., intravenously; DNA may also be administered directly to the target site, e.g., by biolistic delivery to an internal or external target site or by catheter to a site in an artery.

The present invention further relates to a method for the production of a pharmaceutical composition comprising formulating an inhibitor of the interaction of the interacting molecules identified by the method of the invention in a pharmaceutically acceptable form.

The inhibitor may be identified according to conventional protocols. Additionally, molecules that inhibit existing protein-protein interactions can be isolated with the yeast two-hybrid system using the URA3 readout system. Yeast cells that express interacting GAL4ad and LexA fusion proteins which activate the URA3 readout system are unable to grow on selective medium containing 5-FOA. However, when an additional molecule is present in these cells which disrupts the interaction of the fusion proteins the URA3 readout system is not activated and the yeast cells can grow on selective medium containing 5-FOA. Using this method potential inhibitors of a protein-protein interaction can be isolated from a library comprising these inhibitors. Systems corresponding to the URA3 system may be devised by the person skilled in the art on the basis of the teachings of the present invention and are also comprised thereby.

Also, the present invention relates to a method for the production of a pharmaceutical composition comprising identifying a further molecule in a cascade of interacting molecules, of which the at least one member of interacting

molecules identified by any of the above methods is a part of or identifying an inhibitor of said further molecule.

Once at least one member of the interacting molecules has been identified, it is reasonable to expect that said member is a part of a biological cascade. Identification of additional members of said cascade can be effected either by applying the method of the present invention or by applying conventional methods. Also, inhibitors of said further members can be identified and can be formulated into pharmaceutical compositions.

The present invention relates further to a kit comprising at least one of the following:

- (f) host cells as identified in any of the preceding claims and at least one genetic element comprising said genetic information specifying at least one of said possibly interacting molecules containing a counterselectable marker and specified herein above;
- (g) host cells as identified in any of the preceding claims and at least one genetic element not comprising genetic information specifying at least one of said potentially interacting molecules containing a counterselectable marker and specified herein above;
- (h) at least one genetic element comprising said genetic information specifying at least one of said potentially interacting molecules containing a counterselectable marker and specified herein above;
- (i) at least one genetic element not comprising genetic information specifying at least one of said potentially interacting molecules containing a counterselectable marker and specified herein above;

- (j) host cells comprising at least one and preferably at least two of said genetic elements specified in (h) or (i);
- (k) at least one planar carrier carrying nucleic acid or protein from said host cells comprising at least one member of said genetic elements specified herein above wherein said nucleic acid or protein is affixed to said carrier in grid form and optionally solutions to effect hybridization or binding of nucleic acid probes or proteins to said molecules affixed to said grid;
- (l) at least one storage compartment, planar carrier or computer disc comprising or/and characterizing genetic elements, host cells, storage compartments or carriers identified in any of (f) to (k); and/or
- (m) at least one yeast strain comprising a *can1* and a *cyh2* mutation.

Preferably, said kit comprises or also comprises at least one storage compartment containing the host cells of (f), (g) or (j) and/or comprises or also comprises at least one storage compartment containing said genetic information or said potentially interacting molecules encoded by said genetic information as specified in (f) or (h).

The present invention also relates to the use of any of the yeast strains described herein above and in the appended examples for the identification of at least one member of a pair of potentially interacting molecules.

Advantageously, those molecules identified by the method of the present invention as interacting with many different molecules can be recorded. This information can reduce the work needed to further characterise particular interactions since those interactions comprising of a molecule found to

interact with many other molecules within a 2H system may be suspected of being artifactual (Bartel et al., 1993).

Preferably, the data obtained by using the method of the present invention can be accessed through the use of software tools or graphical interfaces that enable to easily query the established interaction network with a biological question or to develop the established network by the addition of further data.

Accordingly, the present invention further relates to a computer implemented method for storing and analysing data relating to potential members of at least one pair or complex of interacting molecules encoded by nucleic acids originating from biological samples, said methods comprising;

- (n) retrieving from a first data-table information for a first nucleic acid, wherein said information comprises;
  - (oa) a first combination of letters and/or numbers uniquely identifying the nucleic acid, and
  - (ob) the type of genetic element comprising said nucleic acid and
  - (oc) a second combination of letters and/or numbers uniquely identifying a clone in which a potential member encoded by said nucleic acid was tested for interaction with at least one other potential member of a pair or complex of interacting molecules
- (p) using said second combination of letters and/or numbers to retrieve from said first data-table or optionally further data-tables, information identifying additional nucleic acids encoding for said at least one other potential member in step (oc).

A preferred embodiment of said method further comprises using said second combination of letters and/or numbers in step f3) to retrieve from a second data-table further information, where said further information at least comprises the

interaction class of said clone, and optionally additional information comprising,

- (q) the physical location of the clone; and
- (r) predetermined experimental details pertaining to creation of said clone, including at least one of:
  - (ra) tissue, disease-state or cell source of the nucleic acid;
  - (rb) cloning details; and
  - (rc) membership of a library of other clones.

It is additionally preferred, that said method comprises using said information of step (o) on said first and/or of step (p) on additional nucleic acids to relate to a third data-table further characterising said first and/or additional nucleic acids, where said further characterising comprises at least one of

- (s) hybridization data,
- (t) oligonucleotide fingerprint data,
- (u) nucleotide sequence,
- (v) in-frame translation of the said nucleic acids, and
- (w) tissue, disease-state or cell source gene expression data; and

optionally identifying the protein domain encoded by said first or additional nucleic acids.

Preferably also said method comprises identifying whether said potential members encoded by the nucleic acids interact, by considering said interaction class of said clone in which nucleic acids were tested for said interaction in step f3).

More preferably, said data relates to one or more of 10 to 100 potential members, yet more preferably 100 to 1000 potential members, yet more preferably, 1000 to 10000 potential members and most preferably more than 10,000 potential members.



In a preferred embodiment, said data was generated by the aforementioned method for identifying members of a pair or complex of interacting molecules.

In a further preferred embodiment, said interaction class comprises one of the following: Positive, or Negative, or False Positive.

It is further preferred, that sticky proteins are identified by consideration of the number of occurrences a given member is identified to interact with many different members in different clones of said positive interaction class.

More preferably, said first data-table forms part of a first database, and said second and third data tables form part of at least a second database.

Yet more preferably, said second database is held on a computer readable memory separate from the computer readable memory holding said first database, and said database is accessed via a data exchange network.

It is further preferred, that said second database comprises nucleic acid or protein sequence, secondary or tertiary structure, biochemical, biographical or gene expression information.

In a particularly preferred embodiment, data entry to said first, second or further data tables is controlled automatically from said first data base by access to other computer data, programs or computer controlled robots.

It is yet more preferred, that at least one workflow management system is built around particular sets of data to assist in the progress of the aforementioned method for identifying members of a pair or complex of interacting molecules.

Most preferably, said workflow management system is software to assist in the progress of the identification of members of a pair or complex of interacting molecules using the aforementioned method of hybridization of nucleic acids.

In another preferred embodiment, said data are investigated by queries of interest to an investigator.

More preferably, said queries include at least one of,

- (aa) identifying the interaction or interaction pathway between a first and second member of an interaction network
- (ab) identifying the interaction pathway between a first and second member of an interaction network and through at least one further member,
- (ac) identifying the interaction or interaction pathway between at least two members characterised by nucleotide acid or protein sequences, secondary or tertiary structures, and
- (ad) identifying interactions or interaction pathways that are different for said different tissue, disease-state or cell source.

Yet more preferably, parts of said information are stored in a controlled format to assist data query procedures.

Even more preferred is a method, wherein the results of said queries are displayed to the investigator in a graphical manner.

Yet more advantageous is the method, wherein a sub-set of data comprising data characterising nucleic acids identified as encoding members of a pair or complex of said interacting molecules is stored in a further data-table or data base.

Yet more preferably, consideration of the number of occurrences a given member is identified to interact with a second or further member is used to decide if said data characterising nucleic acids form part of said sub-set of data.

Even more preferred is the method, wherein additional information or experimental data is used to select those data to form part of said subset.

Most preferably, to speed certain data query procedures, the structure in which the data is stored in the computer readable memory is modified.

In another preferred embodiment, the data is held in relational or object oriented data bases.

The invention further relates to a data storage scheme comprising a data table that holds information on each member of an interaction, where a record in said table represents each member of an interaction, and in which members are indicated to form interactions by sharing a common name.

Preferably, in said data storage scheme said common name is a clone name or unique combination of letters and/or numbers comprising said clone name.

A computer-implemented method for handling of data gathered provides a robust and efficient solution for handling the large amount of protein-protein interaction data produced by the method of the invention. It provides the ability to communicate with and utilise different data-bases and/or other data storage systems across intra or internets, interfaces to allow querying of the data-base by an investigator and visual display of the results of the query. Relational or object orientated data-bases, with data-parsing and display programs supporting said data-base secures ease of use. By way of

example, Figure 2 displays a scheme and features for a set of data-tables suitable for managing such interaction data. The primary links between table-keys are indicated, as are the entry fields or elements to be held within each table. If desired, elements of a table may be expanded into an additional table holding further data. Likewise, certain tables may be expanded into an additional data-base to hold and manage further data. Said additional data-base may be stored on the same or on remote computers. Elements of the table can be recorded in numerical, descriptive or fixed format, whatever is most appropriate for the respective data. To provide efficient querying, where appropriate, elements are recorded in controlled vocabulary. Figure 3 displays in what part of the work process during an interaction experiment each table is most relevant and where it forms the underlying data-set from which work-flow management software for that part of the process is based.

Other computer-based methods of generating visual representations of specific interactions, partial or complete protein-protein interaction networks can be employed to automatically calculate and display the required interactions most efficiently. As is well known in the art, computer data-bases are a valuable resource for large-scale biological and molecular biological research.

In summary, a significant advantage of the method of invention over existing yeast 2H systems is the scale at which such identification of interactions and interaction members can be made. Preferably, the method of invention screens library vs. library interactions using arrayed interaction libraries. Thus, the method of invention allows, in an efficient manner, a more complete and exhaustive generation of protein-protein interaction networks than existing methods. An established and exhaustive network of protein-protein interactions is of use for many purposes as shown in Figure 1. For examples, it may be used to predict the existence of new biological

interactions or pathways, or to determine links between biological networks. Furthermore with this method, the function and localisation of previously unknown proteins can be predicted by determining their interaction partners. It also can be used to predict the response of a cell to changes in the expression of particular members of the networks. Finally, these data can be used to identify proteins or interactions between proteins within a medically relevant pathway which are suitable for therapeutic intervention, diagnosis or the treatment of a disease.

The figures show:

#### Figure 1

The applications of an established and exhaustive network of protein-protein interactions. The identity of positive clones and the identity of the members comprising the interactions for the entire interaction library are stored in a database. These data are used to establish a network of protein-protein interactions which can be used for a variety of purposes. For example, to predict the existence of new biological interactions or pathways, or to determine links between biological networks. Furthermore with this method, the function and localisation of previously unknown proteins can be predicted by determining their interaction partners. It also can be used to predict the response of a cell to changes in the expression of particular members of the networks. Finally, these data can be used to identify proteins within a medically relevant pathway which are suitable for therapeutic, diagnosis intervention and for the treatment of disease.

#### Figure 2

A scheme and features for a set of data-tables suitable for storing, managing and retrieving data from a large-scale protein-protein interaction screen. The scheme could be implemented in either relational or object-orientated databases. The primary links between table-keys are indicated, as

are the suggested fields or elements to be held within each table.

#### Figure 3

A process flow representing the experimental and informatic flow during a large-scale protein-protein interaction screen. The figure displays in which part of the experimental steps each table from a the data-base described above is most applicable. Each table forms the underlying data-set from which work-flow management software for that part of the process is based.

#### Figure 4

Plasmids constructed for the improved 2-hybrid system.

The plasmid maps of the pBTM118a, b and c DNA binding domain vector series and the pGAD428a, b and c activation domain vector series. Both plasmids contain the unique restriction enzyme sites for *Sal* I and *Not* I which can be used to clone a genetic fragment into the multiple cloning site. The plasmids are maintained in yeast cells by the selectable markers TRP1 and LEU2 respectively. The loss of the plasmids can be selected for by the counterselective markers CAN1 and CYH2 respectively.

Polylinkers used within the multiple cloning site to provide expression of the genetic fragment in one of the three reading frames.

#### Figure 5

The structure of the URA3 readout system carried by the plasmid pLUA. Important features of pLUA include the URA3 gene which is under the transcriptional control of the *lexAop*-GAL1 promoter, the *ADE2* selectable marker that allows yeast *ade2*-auxotrophs to grow on selective media lacking adenine and the  $\beta$ -lactamase gene (*bla*) which confers ampicillin resistance in *E.coli*. The pLUA plasmid replicates autonomously both in yeast

using the 2 $\mu$  replication origin and in *E.coli* using the ColE1 origin of replication.

**Figure 6**

A schematic overview of one embodiment of the method of the invention. For the parallel analysis of a network of protein-protein interactions using the method of the invention, a library of plasmid constructs that express DNA binding domain and activation domain fusion proteins is provided. These libraries may consist of specific DNA fragments or a multitude of unknown DNA fragments ligated into the improved binding domain and activating domain plasmids of the invention which contain different selectable and counterselectable markers. Both libraries are combined within yeast cells by transformation or interaction mating, and yeast strains that express potentially interacting proteins are selected on selective medium lacking histidine. The selective markers TRP1 and LEU2 maintain the plasmids in the yeast strains grown on selective media, whereas CAN1 and CYH2 specify the counterselectable markers that select for the loss of each plasmid. HIS3 and lacZ represent selectable markers in the yeast genome, which are expressed upon activation by interacting fusion proteins. The readout system is, in the present case, both growth on medium lacking histidine and the enzymatic activity of  $\beta$ -galactosidase which can be subsequently screened. A colony picking robot is used to pick the resulting yeast colonies into individual wells of 384-well microtiter plates, and the resulting plates are incubated at 30°C to allow cell growth. The interaction library held in the microtiter plates optionally may be replicated and stored. The interaction library is investigated to detect positive clones that express interacting fusion proteins and discriminate them from false positive clones using the method of the invention. Using a spotting robot, cells are transferred to replica membranes which are subsequently placed onto one of each of the selective media SD-leu-trp-his, SD-leu+CAN and SD-trp+CHX. After incubation on the selective plates, the clones which

have grown on the membranes are subjected to a  $\beta$ -Gal assay and a digital image from each membrane is captured with a CCD camera which is then stored on computer. Using digital image processing and analysis clones that express interacting fusion proteins can be identified by considering the pattern of  $\beta$ -Gal activity of these clones grown on the various selective media. The individual members comprising the interactions can then be identified by one or more techniques, including PCR, sequencing, hybridisation, oligofingerprinting or antibody reactions.

#### Figure 7

A schematic overview of one embodiment of the method of the invention. For the parallel analysis of a network of protein-protein interactions with the method of the invention, two libraries of plasmid constructs that express DNA binding domain or activation domain fusion proteins are provided. These libraries may consist of specific DNA fragments or a multitude of unknown DNA fragments ligated into binding domain and activating domain plasmids which contain the selectable markers TRP1 and LEU2, and optionally the counterselective markers CAN1 and CYH2 respectively. The libraries are transformed into either *Mata* or *Mata $\alpha$*  yeast strains containing the URA3 readout system and are subsequently plated onto selective media containing 5-fluoroorotic acid (5-FOA). Only those yeast cells that express fusion proteins unable to auto-activate the URA3 readout system will grow in the presence of 5-FOA. The resulting yeast strains that express only non-auto-activating proteins can then be directly used in an automated interaction mating approach to generate ordered arrays of diploid strains which can be assayed for activation of the lacZ readout system. a) Individual yeast cells that express single fusion proteins unable to activate the URA3 readout system are transferred into wells of a 384-well microtiter plate using a modified picking robot. The yeast strains held in the microtiter plates can optionally be replicated and stored. The microtiter plates contain a growth medium lacking



amino acids appropriate to maintain the corresponding plasmids in the yeast strains. The interaction matings are subsequently performed by automatically transferring a *Mata* and a *Mata $\alpha$*  yeast strain to the same position on a Nylon membrane using automated systems as described by Lehrach et al. (1997). Alternatively, a pipetting or micropipetting system (Schober et al. 1993) can be used to transfer small volumes of individual liquid cultures of a yeast strain onto which a lawn of yeast cells derived from at least one yeast clone of the opposite mating type is sprayed or applied. Yeast strains may be applied singly or as pools of many clones. By both methods ordered arrays of yeast clones are incubated overnight at 30°C to allow interaction mating to occur. The resulting diploid cells are then analysed in a  $\beta$ -Gal assay as described by Breeden & Nasmyth (1985). b) Yeast strains that grew on selective media containing 5-FOA are pooled and interaction mating between the *Mata* and *Mata $\alpha$*  strains is made within liquid YPD medium. Those diploid yeast strains that express interacting proteins are selected by plating on selective medium lacking histidine and uracil. The selective markers TRP1 and LEU2 maintain the plasmids in yeast strains grown on selective media. HIS3, URA3 and lacZ represent reporter genes in the yeast cells, which are expressed on activation by interacting fusion proteins. The readout system is, in the present case, growth on medium lacking histidine and/or uracil and enzymatic activity of  $\beta$ -galactosidase which can be screened at a later time point. A modified colony picking robot is used to pick the diploid yeast colonies into individual wells of 384-well microtiter plates containing selective medium, and the resulting plates are incubated at 30°C to allow cell growth. The interaction library optionally may be replicated and stored. Using a spotting robot, diploid cells are transferred to replica membranes which are subsequently placed onto growth medium. Replica membranes are placed on the counterselective media SD-trp+CHX or SD-leu+CAN. The resulting regular arrays of diploid yeast clones are analysed for  $\beta$ -Gal activity as described by Breeden & Nasmyth

(1985). In either case a) and b), a digital image from each dried membrane is captured with a CCD camera which is then stored on computer. Using digital image processing and analysis clones that express interacting fusion proteins can be identified by considering the  $\beta$ -Gal activity of these clones spotted in a defined pattern grown the membranes placed on the various selective media. The individual members comprising the interactions can then be identified by one or more techniques, including PCR, sequencing, hybridisation, oligofingerprinting or antibody reactions.

#### Figure 8

Predicted interactions between fusion proteins used to create the defined interaction library. The fusion proteins enclosed with dark rounded boxes are believed to interact as shown. The LexA-HIP1 and GAL4ad-LexA fusion proteins enclosed by thin rectangular boxes have been shown to activate the LacZ readout system without the need for any interacting fusion protein. The two proteins LexA and GAL4ad, and the three fusion proteins GAL4ad-HIPCT, GAL4ad-14-3-3 and LexA-MJD (all unboxed) are believed not to interact with each other or other fusion proteins used in this example.

#### Figure 9

Identification of positive clones that contained interacting fusion proteins from false positive clones using the method of the invention. Three different yeast clones each containing pairs of plasmid constructs (positive control: pBTM117c-SIM1 & pGAD427-ARNT; negative control: pBTM117c & pGAD427 and false-positive control: pBTM117c-HIP1 & pGAD427) were transferred by hand to four agar plates each containing a different selective medium (SD-leu-trp, SD-leu-trp-his, SD-leu+CAN and SD-trp+CAN), and incubated for 48 hours at 30 °C. The yeast colonies were subsequently transferred to a Nylon membrane and assayed for  $\beta$ -gal activity by the method of Breeden and Nasmyth (1985).

**Figure 10**

Digital images of the  $\beta$ -gal assays made from the replica Nylon membranes containing the defined interaction library obtained from the selective media (a) SD-leu-trp-his, (b) SD-trp+CHX and (c) SD-leu+CAN. In each case, The left hand side of each membrane contains control clones and clones from the defined interaction library, and the right hand side contains only clones from the defined interaction library. The two regions marked on the first membrane represent those clones magnified in Figure 11. The overall size of each membrane is 22 x 8 cm and contains 6912 spot locations at a spotting pitch of 1.4 mm.

**Figure 11**

Magnification of clones from the interaction library taken from the same region of three membranes obtained from the selective media SD-leu-trp-his, SD-trp+CHX and SD-leu+CAN assayed for  $\beta$ -gal activity:

Clones imaged from a region of the right hand side of the membrane containing the defined interaction library. Clones from the defined interaction library that express interacting proteins are ringed and correspond to the microtiter plate addresses 06L22 and 08N24.

Clones imaged from a region of the left hand side of the same membranes containing control clones and clones from the interaction library, where clones around each ink guide-spot are arranged as shown and correspond to: 00 Ink guide spot; 01 False positive control clone that expresses the fusion protein GAL4ad-LexA; 02 False positive clone expressing the fusion protein LexA-HIP1; 03 Positive control clone expressing the interacting fusion proteins LexA-SIM1 & GAL4ad-ARNT; 04 Clone from the defined interaction library. The positive control clone (spot position 03) is ringed.

**Figure 12**

A subset of the list of clones identified by computer query of data produced by automated image analysis and quantification of the  $\beta$ -galactosidase activity. Each record represents the  $\beta$ -galactosidase activity for a given clone grown on three selective media. This program queried the data to identify all clones from the interaction library that had activated the reporter gene (score > 0) when grown on minimal medium lacking, leucine, tryptophan, and histidine (SD-leu-trp-his), yet had not on either of the counterselective media (score on both media equal to 0).

Two positive clones 06L22 and 08N24 characterised by hybridisation are present within the computer file.

**Figure 13**

Characterisation by hybridisation of the genetic fragments carried by the clones 06L22 and 08N24. A 1.3 kb, SIM1 and a 1.4 kb ARNT DNA fragment were used as nucleic acid probes for hybridisation to high-density spotted membranes containing DNA from the defined interaction library. These clones were characterised as containing SIM1 and ARNT genetic fragments by hybridisation. The images are of the same region of the membranes as those shown in Figure 11 a. The spot positions of the clones 06L22 and 08N24 are ringed.

**Figure 14**

Identification of the SIM1 and ARNT DNA fragments from the yeast two hybrid plasmid carried by the clone 06L22 by duplex PCR. Plasmid DNA was isolated from a liquid culture of the clone 06L22 by a QiaPrep (Hilden) procedure and the inserts contained within the plasmids were amplified by PCR using the primer pairs, 5'-TCG TAG ATC TTC GTC AGC AG-3' & 5'-GGA ATT AGC TTG GCT GCA GC-3' for the plasmid pBTM117c and 5'-CGA TGA TGA AGA TAC CCC AC-3' & 5'-GCA CAG TTG AAG TGA ACT TGC-3' for pGAD427. Lane 1 contains a Lambda DNA digestion with BstEII as size marker; Lane 2 contains the duplex PCR reaction from

plasmids isolated from clone 06L22; Lanes 3 and contain control PCR amplifications from the plasmids pBTM117c-SIM1 and pGAD427-ARNT respectively.

#### Figure 15

Readout system activation for clones in a regular grid pattern from an interaction library. 23 384-well microtiter plates of the sea urchin interaction library were spotted in a '3x3 duplicate' regular grid pattern around an ink guide-spot on a 222 x 222 mm porous membrane (Hybond N+, Amersham, UK) using a spotting robot. The membrane was incubated in SD-leu-trp-his medium for 3 days, assayed for lacZ expression using the  $\beta$ -gal assay as described by Breeden & Nasmyth (1985) and air dried overnight. A digital image was captured using a standard A3 computer scanner.

#### Figure 16

Hybridisation of a gene fragment (Probe A) encoding for Protein A to an array of DNA from an interaction library. The probe was labelled radioactively by standard protocols, and hybridisation-positive clones from the interaction library are identified by the automated image analysis system. The position of clone 5K20, from which the gene fragment was isolated, is indicated. Other hybridisation-positive clones also carry this gene-fragment, and by recovery of interacting members from these clones, a protein-protein interaction pathway for Protein A can be uncovered.

#### Figure 17

A graphical representation of the hybridisation-positive clones generated by hybridisation of Probe A to a DNA array representing the interaction library.

#### Figure 18

A graphical representation of hybridisation- and interaction-positive clones generated by a subsequent hybridisation with probe B (isolated from the clone marked in a grey box). Also

shown, are the positions of the hybridisation-positive clones from probe A. Interaction-positive clones that carry both gene fragments are identified as hybridising with both probes.

#### Figure 19

A graphical representation of hybridisation- and interaction-positive clones generated by a further hybridisation with probe C isolated from the clone 6D18 (marked by a grey box and "B/C"). Also shown are the hybridisation signals for probes A and B. By considering common hybridisation signals for interaction-positive clones and subsequent DNA sequencing of the inserts carried by these clones, protein-protein interactions can be uncovered. The figure also shows an interaction pathway uncovered between Proteins A, B and C based on these data.

#### Figure 20

Automated visual differentiation of yeast cells expressing single fusion proteins able to activate the LacZ readout system. A defined library of L40ccu yeast clones expressing different fusion proteins cloned in the plasmid pBTM117c was plated onto minimal medium lacking tryptophan, buffered to pH 7.0 with potassium phosphate and containing 2 ug/ml of X-Gal (SD-trp/XGAL). White colonies that have not autoactivated the LacZ reporter gene are automatically recognised and marked with a red horizontal cross. A colony that has turned blue due to expression of a single fusion protein able to auto-activate the LacZ reporter gene is automatically recognised due to its darker colour and the presence of a 'hole'. An arrow indicates this colony. All colonies unsuitable for further analysis and picking (including those too small or touching colonies) are automatically recognised and marked with a blue diagonal cross.

#### Figure 21

Results of automated interaction mating to identify diploid yeast strains that express interacting fusion proteins. a)

Progeny of the yeast strains x1a and x2a were spotted at positions 1 and 2 on a nylon membrane using a spotting robot such as described by Lehrach et al. (1997). The yeast strains y1a and y2a of the opposite mating type were subsequently spotted on positions 1 and 2 which already contained cells from the strains x1a and x2a. To assist in recognition of the duplicate spotting pattern, ink was spotted in position 2 directly to the right of the spotted yeast clones. b) The membrane was transferred to a YPD agar plate and was incubated at 30° C overnight to allow interaction mating to occur. c) Diploid yeast cells that had grown on the membrane were subsequently analysed for  $\beta$ -galactosidase activity using the method of Breeden & Nasmyth (1985).

#### Figure 22

The two vectors constructed to provide further genetic features to enable the method of invention within a prokaryotic two-hybrid system. The vectors are based on the pBAD series of vectors which provide tight inductive-control of expression of cloned genes using the promoter from the arabinose operon (Guzman et al., 1995 J. Bact. 177: 4141-4130), and can be maintained in the same *E.coli* cell by virtue of compatible origins of replication.

The plasmid pBAD18- $\alpha$ RNA<sub>P</sub> expresses under the control of the arabinose promoter, fusion proteins between the  $\alpha$  amino terminal domain (NTD) of the  $\alpha$ -subunit of RNA polymerase and DNA fragments cloned into the multiple cloning site. The presence of this plasmid in kanamycin sensitive cells can be selected by plating on growth medium supplemented with kanamycin, or for its absence by the counterselective *rpsL* allele by plating on media supplemented with streptomycin (Murphy et al. 1995).

The plasmid pBAD30-cI expresses under the control of the arabinose promoter, fusion proteins between the  $\lambda$ cI protein and DNA fragments cloned into the multiple cloning site. The

presence of this plasmid in ampicillin sensitive cells can be selected by plating on growth medium supplemented with ampicillin, or for its absence by the counterselective *lacY* gene by plating on media supplemented with 2-nitrophenyl- $\beta$ -D-thiogalactosidase (tONPG) (Murphy et al. 1995). Additionally, the *oriT* sequence enables unidirectional genetic exchange of the pBAD30-cI plasmid and its derivatives from *E. coli* cells containing the F' fertility factor to F<sup>-</sup> strains lacking the fertility factor.

### Examples

**Example 1:** Construction of vectors yeast strains and readout system for an improved yeast two-hybrid system

#### 1.1 Construction of vectors

The plasmids constructed for an improved yeast two-hybrid system pBTM118 a, b and c and pGAD428 a, b and c are shown in Figure 4. Both sets of vectors can be used for the construction of hybrid (fusion) proteins. The vectors contain the unique restriction sites *Sal* I and *Not* I located in the multiple cloning site (MCS) region at the 3'- end of the open reading frame for either the *lexA* coding sequence or the *GAL4ad* sequence Figure 4b).

With both sets of plasmids fusion proteins are expressed at high levels in yeast host cells from the constitutive *ADH1* promoter (P) and the transcription is terminated at the *ADH1* transcription termination signal (T). The two-hybrid plasmids shown in Figure 4a are shuttle vectors that replicate autonomously in both *E. coli* and *S. cerevisiae*.

The three plasmids pBTM118 a, b and c are used to generate fusions of the LexA protein (amino acids 1-220) and a protein of interest cloned into the MCS in the correct orientation and



reading frame. The plasmids pBTM118 a, b and c are derived from pBTM117c (Wanker et al., 1997) by insertion of the adapters shown in Table 1 into the restriction sites *Sal* I and *Not* I to generate the improved vectors with three different reading frames.

The plasmids pBTM118 a, b and c carry the wild type yeast *CAN1* gene for counterselection, which confers sensitivity to canavanine in transformed yeast cells (Hoffmann, 1985). The plasmids also contain the selectable marker *TRP1*, that allows yeast *trp1*-auxotrophs to grow on selective synthetic medium without tryptophan, and the selectable marker *bla* which confers ampicillin resistance in *E. coli*.

The plasmids pGAD428 a, b and c are used to generate fusion proteins that contain the GAL4 activation domain (amino acids 768-881) operatively linked to a protein of interest. The plasmids pGAD428 a, b and c carry the wild type yeast *CYH2* gene, which confers sensitivity to cycloheximide in transformed cells (Kaeufer et al., 1983), the selectable marker *LEU2*, that allows yeast *leu2*-auxotrophs to grow on selective synthetic medium without leucine, and the bacterial marker *aphA* (Pansegrau et al., 1987) which confers kanamycin resistance in *E. coli*. The plasmids pGAD428a, b and c were created from pGAD427 by ligation of the adapters shown in Table 1 into the MCS to construct the improved vectors with three different reading frames.

For the construction of pGAD427 a 1.2 kb *Dde* I fragment containing the *aphA* gene was isolated from pFG101u (Pansegrau et al., 1987) and was subcloned into the *Pvu* I site of the pGAD426 using the oligonucleotide adapters 5'-GTCGCGATC-3' and 5'-TAAGATCGCGACAT-3'. The plasmid pGAD426 was generated by insertion of a 1.2 kb *Eco* RV *CYH2* gene fragment, which was isolated from the pAS2-1 (Clontech) into the *Pvu* II site of pGAD425 (Han and Collicelli, 1995).

## 1.2 Construction of yeast strains

To allow for the improved yeast two-hybrid system, three *Saccharomyces cerevisiae* strains L40cc, L40ccu and L40ccu $\alpha$  were created. The *S. cerevisiae* L40cc was created by site specific knock-out of the CYH2 and CAN1 genes of L40 (Hollenberg et al., Mol. Cell. Biol. 15: 3813-3822), and L40ccu created by site specific knock-out of the URA3 gene of L40cc (Current Protocols in Molecular Biology, Eds. Ausubel et al. John Wiley & Sons: 1992) The strain L40ccu $\alpha$  was created by conducting a mating-type switch of the strain L40ccu by standard procedures (Ray BL, White CI, Haber JE (1991)). The genotype of the L40cc strain is: *Mata his3 $\Delta$ 200 trp1-901 leu2-3,112 ade2 LYS2::(lexAop)<sub>4</sub>-HIS3 URA3::(lexAop)<sub>8</sub>-lacZ GAL4 can1 cyh2*, The genotype of the L40ccu strain is: *Mata his3 $\Delta$ 200 trp1-901 leu2-3,112 ade2 LYS2::(lexAop)<sub>4</sub>-HIS3 ura3::(lexAop)<sub>8</sub>-lacZ GAL4 can1 cyh2*, and that of L40ccu $\alpha$  is *Mata his3 $\Delta$ 200 trp1-901 leu2-3,112 ade2 LYS2::(lexAop)<sub>4</sub>-HIS3 ura3::(lexAop)<sub>8</sub>-lacZ GAL4 can1 cyh2*.

## 1.3 Readout system

Figure 5 shows the URA3 readout system carried by the plasmid pLUA. This URA3 readout system under the control of a bacterial LexAop upstream activation sequence (UAS) can be used within the yeast 2-hybrid system both as a counter selective reporter gene and as a positive selection reporter gene to eliminate false positive clones. The plasmid contains the features of the UAS<sub>lexAop</sub>-URA3 readout system, the selectable marker ADE2 that allows yeast ade2-auxotrophs to grow on selective media without adenine and the bla gene which confers ampicillin resistance in *E.coli*. The plasmid pLUA is a shuttle vector that replicates autonomously in *E. coli* and yeast.

For the construction of pLUA a 1.5 kb Sac I/Cla I UAS<sub>lexAop</sub>-URA3 fragment was isolated from pBS-lexURA and ligated

together with a 2.4 kb *Sac* I/*Cla* I *ADE2* fragment into *Cla* I digested pGAD425Δ. pBS-lexURA was generated by ligating *URA3* fragment together with a UAS<sub>lexAop</sub> fragment into pBluescript SK+. The *URA3* and UAS<sub>lexAop</sub> fragments were obtained by PCR using genomic DNA from *S. cerevisiae* strain L40c using standard procedures and anchor primers which gave rise to complementary overhangs between the two consecutive fragments which were subsequently annealed to generate the chimeric sequence (see, for example, Current Protocols in Molecular Biology, Eds. Ausubel et al. John Wiley & Sons: 1992). The *ADE2* gene was isolated by PCR using genomic DNA from SEY6210α. pGAD425Δ was generated by deleting of an 1.2 kb *Sph* I fragment from pGAD425 (Han and Colicelli, 1995) and religation of the vector.

#### 1.4 Generation of a defined interaction library

To determine if the invention could be used in an improved two-hybrid system for yeast, as shown in Figure 6 or Figure 7, a defined interaction library of plasmids that express various LexA and GAL4ad fusion proteins of interest was constructed using the vectors and strains described in sections 1.1 and 1.2. The orientation of the inserted fragments was determined by restriction analysis and the reading frame was checked by sequencing. The generated constructs and the original plasmids described above are listed in Table 2. The construction of pBTM117c-HD1.6, -HD3.6 and -SIM1 was described elsewhere (Wanker et al., 1997; Probst et al., 1997). pBTM117c-HIP1 and pGAD427-HIP1 were obtained by ligation of a 1.2 kb *Sal* I HIP1 fragment isolated from pGAD-HIP1 (Wanker et al., 1997) into pBTM117c and pGAD427, respectively. pBTM117c-MJD was created by inserting a 1.1 kb *Sal* I/*Not* I MJD1 fragment (Kawagushi et al., 1994) into pBTM117c, and pGAD427-14-3-3 was generated by inserting a 1.0 kb *Eco*RI/*Not*I fragment of pGAD10-14-3-3 into pGAD427. For the construction of pGAD427-HIPCT, a 0.5 kb *Eco*RI HIP1 fragment isolated from pGAD-HIPCT (Wanker et al., 1997) was ligated into pGAD427. pGAD427-lexA and pGAD427-ARNT

were generated by insertion of a 1.2 kb *Sal* I/Not digested *lexA* PCR fragment and a 1.4 kb *Sal* I/Not I *ARNT* fragment into *pGAD427* respectively.

It was shown that the fusion proteins *LexA-SIM1* and *GAL4ad-ARNT* specifically interact with each other in the yeast two-hybrid system (Probst et al., 1997), because when both hybrids were coexpressed in *Saccharomyces cerevisiae* containing two integrated reporter constructs, the yeast *HIS3* gene and the bacterial *lacZ* gene, which both contained binding sites for the *LexA* protein in the promoter region, the interaction between these two fusion proteins led to the transcription of the reporter genes. The fusion proteins by themselves were not able to activate transcription because *GAL4ad-ARNT* lacks a DNA binding domain and *LexA-SIM1* an activation domain (Probst et al., 1997). In contrast it was shown recently that the fusion proteins *LexA-HIP1* and *GAL4ad-LexA* are capable of activating the *HIS3* and *lacZ* reporter genes without interacting with a specific *GAL4ad* or *LexA* fusion protein respectively. Thus, the yeast clones expressing the *LexA-HIP1* protein have to be designated as false positives, because false positives are defined here as clones where a *GAL4ad* fusion protein or a *LexA* fusion protein alone without the respective partner protein activates the transcription of the reporter genes without the need for any interacting partner protein.

The predicted protein-protein interactions of these fusion proteins are shown in Figure 8. It was shown that the fusion proteins *LexA-SIM1* & *GAL4ad-ARNT*, *LexA-HD1.6* & *GAL4ad-HIP1* and *LexA-HD3.6* & *GAL4ad-HIP1* specifically interact with each other in the yeast two-hybrid system because they only activate the reporter genes *HIS3* and *lacZ* when both proteins are present in one cell (Probst et al. 1997; Wanker et al. 1997). In contrast, it was demonstrated that the *LexA-HIP1* and *GAL4ad-LexA* fusion proteins are capable of activating the reporter genes without the need for any interacting fusion protein. The proteins *LexA* and *GAL4ad* and the fusion proteins *LexA-MJD* and

GAL4ad-14-3-3 which are also present in the defined interaction library are unable to activate the reporter genes either alone or when present in the same cell with any other fusion proteins comprising the library.

**Example 2:** Detection of clones expressing known interacting proteins from false positives using the improved two-hybrid system

Pairs of the yeast two-hybrid plasmids pBTM117cSIM1 & pGAD427-ARNT, pBTM117c & pGAD427 and pBTM117c-HIP1 & pGAD427 were transformed into the yeast strain L40cc, and Trp+Leu+ transformants that contained at least one of each of the two plasmids were selected on SD-leu-trp plates. Two transformants from each transformation were investigated for the presence of protein-protein interactions by testing the ability of the yeast cells to grow on SD-leu-trp, SD-leu-trp-his, SD-leu+CAN and SD-trp+CHX plates and by the  $\beta$ -galactosidase membrane assay (Breen and Nasmyth, 1985). Figure 9 shows that the yeast strains cells harboring both the plasmids pBTM117c-SIM1 & GAD427-ARNT or pBTM117c-HIP1 & pGAD427 grow on SD-leu-trp-his plates and turned blue after incubation in X-Gal solution, indicating that the *HIS3* and *lacZ* reporter genes are activated in these strains. In comparison, the yeast strain harboring both the negative control plasmids pBTM117c & pGAD427 was not able to grow on this medium and also showed no *lacZ* activity. After selection of the yeast strains harboring the different combinations of the two-hybrid plasmids on SD-leu+CAN and SD-trp+CHX the resulting strains were also analyzed by the  $\beta$ -galactosidase assay. After incubating the membrane containing all three strains on SD-trp+CHX medium only progeny of the yeast strain that originally harbored both the plasmids pBTM117c-HIP1 & pGAD427 yet which had lost the pGAD427 plasmid through counterselection turned blue after incubating in X-Gal solution. This result indicates that this clone is a false positive, because although showing a *lacZ*+ phenotype when grown on SD-leu-trp-his medium, the LexA-HIP1 fusion protein

was also capable of activating the HIS3 and lacZ genes on SD-trp+CAN medium without the need for any interacting fusion protein. In comparison, the yeast strain harboring both the plasmids pBTM117c-SIM1 & pGAD427-ARNT is a positive clone that expresses interacting LexA and GAL4ad fusion proteins, because both the LexA and the Gal4ad fusion proteins are necessary for the activation of the reporter genes. If either of the plasmids pBTM117c-SIM1 or pGAD427-ARNT is lost from the strain by counterselection on SD-trp+CHX or SD-leu+CAN, respectively, the resulting cells are no longer able to activate the lacZ reporter gene and do not turn blue after incubation in X-Gal solution. With the membranes from the SD-leu+CAN plate false positive clones expressing an auto-activating GAL4ad-LexA fusion protein were also detected by the  $\beta$ -galactosidase assay.

**Example 3:** Generation of regular grid patterns of host cells expressing potentially interacting fusion proteins

### 3.1 Generation of a regular grid pattern of clones from an interaction library in microtiter plates using automation

To generate the well defined interaction library, the constructs for the expression of the fusion proteins shown in Figure 8 were pooled and 3  $\mu$ g of the mixture was co-transformed into yeast strain L40cc by the method of Schiestel & Gietz (1989). The yeast cells co-transformed with the constructs described in Table 2 were plated onto large 24 x 24 cm agar trays (Genetix, UK) containing minimal medium lacking tryptophan leucine and histidine (SD-leu-trp-his). The agar trays were poured using an agar-autoclave and pump (Integra, Switzerland) to minimise tray-to-tray variation in agar colour and depth. To maximise the efficiency of automated picking, the transformation mixture were plated such that between 200 and 2000 colonies per agar tray were obtained after incubation at 30°C for 4 to 7 days.

Suitable changes to the hardware and software of a standard picking robot designed for the picking of *E. coli* cells as described by Lehrach et al. (1997) were made to account for the specific requirements of yeast cells. The illumination of agar-trays containing plated colonies was changed from the dark-field sub-illumination to dark-field top-illumination to differentiate yeast colonies from the lawn of non-transformed cells. The existing vision guided motion system (Krishnaswamy & Agapakis 1997) was modified to allow for a larger range of 'blob' size when selecting yeast colonies to pick from the blob-feature-table returned by connectivity algorithms when applied to a digital image of the agar tray containing colonies. The clone inoculation routine was re-programmed to ensure that cell material which had dried on the picking pins during the picking routine was initially re-hydrated by 10 seconds of immersion in the wells of a microtiter plate before vigorous pin-motion within the well. This robotic procedure ensured that sufficient cell material was inoculated from each picking pin into an individual well of a microtiter plate. The picking pins were sterilised after inoculation to allow the picking cycle to be repeated by programming the robot to brush the picking pins in a 0.3% (v/v) solution of hydrogen peroxide, followed by a 70% ethanol rinse from a second wash-bath and finally drying by use of a heat-gun to evaporate any remaining ethanol from the pins. Furthermore, an algorithm to automatically correct for height variation in the agar was incorporated by referencing the surface height of the agar in three corners and from these points automatically estimating the surface plane of the agar. The robot was further programmed to automatically adjust both the imaging and picking heights according to the agar surface height such that when a pin was extended into a colony, it removed cells only from the top surface of the colony and did not penetrate the whole colony into the growth medium. Finally, we incorporated additional selection criteria that would reliably sort between blue and white colonies. Although the robot provided a method to select only those 'blobs' (colonies) within a range of

average grey scales (eg, > 80 for white colonies), this proved unreliable since the actual value of average grey scale required to make a correct discrimination varied across the agar-tray due to slight variation in intensity of the illumination. Therefore, a new method was implemented that automatically modified this discrimination value based on the average illumination of a region of the agar-tray as measured by the camera on a frame-to-frame basis. Often, a 'blue' colony that activated the readout system was not uniformly blue across the its whole area, but only the centre would be blue and the surrounding cell material was white. In such cases, the connectivity algorithms would detect two 'blobs' - one (the blue centre) lying directly on the other (the white surrounding) and although the former would be ignored since it was blue, the latter would be selected as its average grey-scale was greater than the discrimination value. Such cases were successfully selected against by ignoring any colonies that had 'holes' using a 'number of holes' function of the image analysis program, which flags those blobs which have a second blob within their boundary.

Using these modifications to a laboratory picking robot, individual yeast colonies were automatically picked from the agar-trays into individual wells of a sterile 384-well microtiter plate (Genetix, UK) containing sterile liquid minimal medium lacking leucine and trptophan (SD-leu-trp) and containing 7% (v/v) glycerol. The resulting microtiter plates were incubated at 30°C for 36 hours, the settled colonies were dispersed by vigorous mixing using a 384-well plastic replicating tool (Genetix, UK) and then incubated for a further 2 to 4 days. A picking success of over 90% wells containing a growing yeast culture was achieved. After growth of yeast strains within the microtiter plates, each plate was labelled with a unique number and barcode. Each plate was also replicated to create two additional copies using a sterile 384-pin plastic replicator (Genetix, UK) to transfer a small amount of cell material from each well into pre-labelled 384-



well microtiter plates and pre-filled with SD-leu-trp-his/7% glycerol liquid medium. The replicated plates were incubated at 30 °C for 3 days with a cell dispersal step after 36 hours, subsequently frozen and stored at -70°C together with the original picked microtiter plates of the interaction library.

In this manner, a regular grid pattern of yeast cells expressing potentially interacting yeast clones was generated using a robotic and automated picking system. 384-well microtiter plates have a well every 4.5 mm in a 16 by 24 well arrangement. Therefore, for each 384-well microtiter plate a regular grid pattern at a density greater than 4 clones per square centimetre was automatically created.

### 3.2 Creation of regular grid patterns of increased density

To generate arrays with higher densities, a computer-controlled 96-well pipetting system (Opal-Jena) with automatic plate-stacking, tip washing, liquid waste and accurate x-y positioning of the microtiter plate currently accessed by the tips was employed. The yeast two hybrid cells that had settled in the bottom of the wells of the arrayed interaction library as described above were re-suspended, and a stack of these 384-well plates were placed into the input stacker of the pipetting system. The system was programmed to take a single 384-well microtiter plate containing the arrayed yeast two-hybrid clones and parallel aspirate 10  $\mu$ l of culture medium and cells into each of the 96 pipette tips from 96 wells of the 384-well plate. The inter-tip spacing of the 96 tips was 9mm and the wells of the 384-well microtiter plate were 4.5 mm so that cells were removed from only every other well along each dimension of the 384-well plate. 8  $\mu$ l of the 96 aspirated samples contained in the tips were then pipetted in parallel into one set of wells of a sterile 1536-well microtiter plate (Greiner, Germany). Since the inter-well spacing of this 1536-well microtiter plate is 2.25 mm, yeast cells were deposited into only 1 every 4 wells along each dimension of the 1536-

well plate. The remaining 2  $\mu$ l of culture medium and cells was aspirated to waste before sterilising each 96 tips in parallel. Sterilisation was conducted by twice aspirating and washing to waste 50  $\mu$ l of 0.3% (v/v) hydrogen peroxide stored in a first replenishable wash-bath on the system, and then aspirating and washing to waste 50  $\mu$ l sterile distilled water stored in a second replenishable wash-bath.

This plate-to-plate pipetting cycle was repeated 3 further times, each time aspirating a different set of 96-clones from the 384-well array of yeast 2-hybrid clones into a different set of 96-wells in the 1536-well microtiter plate by moving the microtiter plates relative to the 96-tips using the accurate x-y positioning of the system. When all clones of the first 384-well microtiter plate had been sampled and arrayed into the 1536-well plate, the first 384-well microtiter plate was automatically exchanged for the next 384-well microtiter plate, and the yeast 2-hybrid clones arrayed in this second 384-well plate were similarly arrayed into the 1536-well plate. When the yeast 2-hybrid clones contained within four 384-well microtiter plates had been automatically arrayed in the first 1536-well plate, filling all wells, the 1536-well plate was automatically exchanged for a second sterile 1536-well plate stored in the second stacking unit of the pipetting system. The whole process was repeated until all yeast 2-hybrid clones of the interaction library had been automatically transferred from 384-well to 1536-well microtiter plates.

In this manner, a regular grid pattern of yeast cells expressing potentially interacting yeast clones using a computer-controlled pipetting system was generated. 1536-well microtiter plates have a well every 2.25 mm in a 32 by 48 well arrangement. Therefore, for each 1536-well microtiter plate we automatically created a regular grid pattern at a density greater than 19 clones per square centimetre.

### 3.3 Generation of a regular grid pattern of clones from an interaction library on porous carriers using automation

A high-throughput spotting robot such as that described by Lehrach et al. (1997) was used to construct porous planar carriers with a high-density regular grid-pattern of yeast clones from the defined interaction library contained within 384-well microtiter plates. The robot recorded the position of individual clones in the high-density grid-pattern by the use of a pre-defined duplicate spotting pattern and the barcode of the microtiter plate. Individually numbered membrane sheets sized 222 x 80 mm (Hybond N+, Amersham UK) were pre-soaked in SD-leu-trp-his medium, carefully laid on a sheet of 3mm filter paper (Whatmann) pre-soaked in the same medium and placed in the bed of the robot. The interaction library was automatically arrayed as replica copies onto the membranes using a 384-pin spotting tool affixed to the robot. Five different microtiter plates from the first copy of the interaction library were replica spotted in a '3x3 duplicate' pattern around a central ink guide-spot onto 10 nylon membranes - corresponding to approximately 1900 clones spotted at a density of approximately 40 spots per cm<sup>2</sup>. On each replica membrane three different control clones were spotted, each from a microtiter plate that contained the same control clone in every well. One control clone expressed the fusion proteins LexA-SIM1 & GAL4ad-ARNT, a second control clone the fusion protein LexA-HIP1, while a third expressed fusion protein GAL4ad-LexA, and all were spotted in order to test the selection, counterselection and the  $\beta$ -gal assay features of the method. To ensure the number of yeast cells on each spot was sufficient for those membranes which were to be placed on the counterselection media plates, the robot was programmed to spot onto each spot position 5 times from a slightly different position within the wells of the microtiter plates. The robot created a data-file in which the spotting pattern produced and the barcode that had been automatically read from each microtiter plate was recorded.

Each membrane was carefully laid onto approximately 300 ml of solid agar media in 24 x 24 cm agar-trays. Six membranes were transferred to SD-leu-trp-his media and two each of the remaining membranes were transferred to either SD-trp+CHX or SD-leu+CAN media. The yeast colonies were allowed to grow on the surface of the membrane by incubation at 30 °C for 3 days.

#### 3.4 Generation of a regular grid pattern of clones from an interaction library on non-porous carriers using automation

The plasmid pGNG1 (MoBiTec, Germany) carries a green fluorescent protein variant under the control of a LexA operator. This variant, GFPuv, is up to 16 times brighter than the wild-type variant isolated from *Aequora victoria* (Ausubel et al., 1995; Short protocols in molecular biology, 3<sup>rd</sup> ed. John Wiley & Sons, New York, NY.). The yeast 2 $\mu$ m origin of replication and the auxotrophic marker URA3 maintains the plasmid in ura3 mutant yeast strains. This plasmid should act as a readout system to detect single fusion proteins or interacting fusion proteins able to activate the readout system in the method of invention described herein. As is known in the art, green fluorescent proteins and its variants are considered suitable reporter genes in most host-cell types. Therefore, it would be possible for a person skilled in the art to incorporate this gene within other host-cell types and interaction systems as disclosed in this invention.

The yeast strain L40ccu was transformed with the plasmid pGNG1 (MoBiTec, Germany) using the method of Schistel & Gietz (1989), and a resulting stable transformant clone cultured in minimal medium lacking uracil and subsequently used to generate two further yeast clones, each containing two genetic elements. The first strain, GNGp, was generated by co-transformation of a mixture of the plasmids pBTM117c-SIM1 and pGAD427-ARNT co-transformed into L40ccu already carrying the reporter plasmid pGNG1. The second strain, GNGn, was generated

by co-transformation of a mixture of the plasmids pBTM117c-MJD and pGAD427-14-3-3 co-transformed into L40ccu already carrying the reporter plasmid pGNG1. In both cases, the transformations were conducted using the method of Schistel & Gietz (1989), and transformants were selected by plating on minimal media lacking uracil, tryptophan and leucine.

Individual colonies from the two transformations were picked into individual wells of 384-well microtiter plates as described in section 3.1 except that the microtiter plates contained liquid minimal medium lacking uracil, tryptophan and leucine. One microtiter plate was created that contained individual colonies of the GNGp yeast strain, and another carrying colonies of GNGn. Using a spotting robot (Lehrach et al., 1997) fitted with high precision spotting tool carrying 16 pins in a 4 x 4 pattern, the clones were arrayed onto polylysine coated glass-slide (Sigma, US). The clones were spotted at a spacing of 440  $\mu$ m, with a spot diameter of approximately 300  $\mu$ m generating a density of over 490 clones per square centimetre. To increase the amount of cell material deposited at each spot, the robot was programmed to spot onto each spot position 10 times from a slightly different position within the wells of the microtiter plates. It is well known in the art that piezo-ink-jet micropipetting systems (Kietzmann et al., 1997, Schober et al., 1993) can create regular grid pattern of clones at an even greater density. Indeed, grid densities of over 1600 spots per square centimeter have been achieved with such systems.

The fluorescent readout system of cells in the regular grid pattern of cells was then visualised using a sensitive CCD camera (LAS1000, Fuji, Japan). Appropriate excitation light was provided and an emission filter appropriate for the emission spectrum of GFP<sub>uv</sub> was fitted to the lens. Other imaging systems could be utilised to investigate the regular grid pattern of clones. For example, laser-scanning systems including laser scanning confocal microscopes would be

preferred when imaging very high density regular grid patterns, or for those formed from a small number of host cells deposited at each position.

It was shown that the fusion proteins LexA-SIM1 and GAL4ad-ARNT can interact and activate a readout system under control of the LexA operator. Since the GNG<sub>UV</sub> reporter gene is under the control of a LexA operator, a cell carrying the pGNG1 plasmid and expressing these fusion proteins should fluoresce under UV light. In contrast, the fusion proteins LexA-MJD and GAL4-14-3-3 were shown unable to activate the same readout system. Image analysis of the digital image of the regular grid pattern of yeast cells, demonstrated that indeed, the GNGp yeast strain did fluoresce while the GNGn did not.

As an alternative to pGNG1 a person skilled in the art could subclone an improved GFP mutant as described in Anderson et al. (1996). Replacement of the URA coding sequence in pLUA (section) with GFP is performed by using appropriate anchor primer to amplify the GFP mutant. Using the appropriate growth media the analysis can be performed as described above.

**Example 4:** Detection of the readout system in a regular grid pattern.

4.1 Detection of readout system activation in a regular grid pattern of clones from an interaction library on planar carriers using digital image capture, processing and analysis

Two membranes from each of the selective media described in section 3.3 were assayed for lacZ expression using the  $\beta$ -gal assay as described by Breeden & Nasmyth (1985) and air dried overnight. For each membrane, a 24-bit digital BMP (bitmap) image of the  $\beta$ -gal assay was captured using a standard A3 computer scanner, and the images were stored on computer. The yeast strain used to create the defined interaction library was an ade2 auxotrophic mutant, and those colonies that grew

yet did not activate the readout system were pink in colour when mature. Since image analysis programs used for the analysis of DNA grids use single channel (grey-scale) images, it was necessary to convert this colour image to an 8-bit grey-scale image. However, the pink colour of colonies not expressing the  $\beta$ -gal reporter gene, when converted to grey-scale, would lower the contrast between positive and negative activation states of the readout system. Therefore, the pink-red colours of the image were re-mapped to light yellow before processing the remapped 24-bit colour image to a colour-inverted 8-bit grey-scale TIF (tagged image file format) using the software Photo Magic (Micrografix, USA). One non-inverted 8-bit grey-scale image of the defined interaction library that was grown on membranes placed on each of the 3 selective media and subsequently assayed for  $\beta$ -gal activity is shown in Figure 10.

Individual clones of the interaction library can be identified and their position on the high-density spotted filter converted to specific wells in the microtiter plates using an automated image analysis system as described by Lehrach et al. (1997). Here, the basic grid and node position of each clone is determined through an iterative sampling scheme proposed by Geman & Geman (1984). Once the node positions have been determined, the average grey-scale value of a pixel mask appropriately sized for the average colony diameter is recorded from the image for every colony on the filter. From these intensity data, global and block-specific background corrections are made, giving greater weight to the local block-specific background. Each colony is then classified into one of four  $\beta$ -galactosidase activities by appropriate binning values of the background-corrected intensities.

Positive clones that expressed interacting fusion proteins were detected from false positive clones by considering the activity of  $\beta$ -galactosidase of clones grown on spotted membranes laid on the various selective media. Positive clones

should activate the lacZ reporter gene on SD-leu-trp-his media and turn blue on incubation with X-Gal solution, but not on either of the two counterselective media. False positive clones should activate the reporter gene and turn blue on incubation with X-Gal solution on at least one counterselective media as well as on the SD-leu-trp-his medium.

Figure 11 shows magnified images of a  $\beta$ -gal assay of clones grown on the membranes which had been placed on the three selective media. Within the magnified region of the membranes shown in Figure 11a, two clones were detected as positive clones that express interacting fusion proteins since they activated the lacZ reporter gene on SD-leu-trp-his media, but not on either of the two counterselective media, and whose spotted positions are circled. The two clones were identified by their microtiter plate address within the interaction library as 06L22 and 08N24 respectively. All other clones spotted within this region of the membrane were detected as false positive since they express  $\beta$ -galactosidase on SD-trp+CHX medium as well as on SD-leu-trp-his medium.

Expression of the LacZ reporter gene for the three control clones spotted onto the same membranes confirm these results. The positive control clone that expresses the interacting fusion proteins LexA-SIM1 & GAL4ad-ARNT should show a LacZ+ phenotype when grown on SD-leu-trp-his medium, but LacZ- when grown on either of the counterselective media. This control clone was spotted at position 03 in the region of the membranes shown in Figure 11b, of which one example is circled. The pattern of  $\beta$ -gal activity for this positive control clone on the three selective media is as predicted. The false positive control clone that expresses the fusion protein LexA-HIP1 and the false positive clone that expresses the fusion protein GAL4ad-LexA are spotted at positions 02 and 01 respectively. Both false positive control clones show a LacZ+ phenotype when grown on SD-leu-trp-his media, but are



detected as false positive clones by the method of the invention since they also show a LacZ+ phenotype on SD-leu+CAN or SD-trp+CHX media, respectively. The clones spotted at position 04 are from the defined interaction library, and from their LacZ+ phenotype when grown on SD-leu+CAN media are predicted to be false positive clones.

The image analysis system described above was used to automatically identify those individual clones on each high-density regular grid pattern that had activated the LacZ readout system. This was conducted for each of the membranes grown on the three selective media, and the intensity of  $\beta$ -galactosidase activity for each clone grown on the three media was automatically recorded by the program using a scale from 0 to 3 (no activity, weak activity, medium activity, high activity). These data for all clones on a given membrane were saved in a computer file, and the  $\beta$ -galactosidase activity for a given clone was related to its activity when grown on the other two selective media using a computer program. This program was used to query and identify all clones from the interaction library that had activated the reporter gene when grown on SD-leu-trp-his (score greater than 0), yet had not on either of the counterselective media (score on both media equal to 0). Figure 12a shows a subset of these clones identified using this data-query procedure, and Figure 12b shows that the two clones 06L22 and 08N24 are found within this automatically identified data-set of positive clones.

4.2 Detection of readout system activation in a regular grid pattern of clones from an interaction library in microtiter plates using digital image capture, processing and analysis.

The interaction library comprising the yeast cells as described in section 3.1 were screened in microtiter plate format to identify those cells that express interacting fusion proteins. First, microtiter plates containing the interaction library were removed from frozen storage and thawed to room

temperature. Second, each plate was replicated and labelled as described in section 3.1 to create additional copies for screening, each into 3 separate selective media. Cells were transferred into 384-well microtiter plates pre-filled with 40  $\mu$ l of the liquid selective media SD-leu-trp, SD-leu+Can or SD-trp+CHX. Third, after growth for 4 days at 30°C, 10  $\mu$ l of Yeast One Step Yeast Lysis Buffer containing Galacton-Star and Sapphire II (Tropix, US) was added, the cells were dispersed using a plastic replication tool, and the plates incubated for 40 min at 37°C. Finally, a digital image of six plates was obtained in parallel using a LAS1000 CCD camera (Fuji, Japan), by placing the plates side-by-side in a two by three arrangement. The  $\beta$ -galactisidase substrate, Galacton-Star in combination with Sapphire II (Tropix, US) generates detectable luminescent light on activation of the  $\beta$ -gal reporter gene in the yeast-cells, and an exposure time of 5 minutes was used to collect sufficient signal. The grey-scale digital images were captured, saved on computer and subsequently analysed using the image analysis system described in section 4.1. However, in this case, the position of each clone was far simpler to determine due to the lower density of the regular grid pattern of clones in the microtiter plate. Second, the size of the pixel mask used to measure the average pixel intensity was approximately that of the size of the microtiter plate well. Positive clones in the six microtiter plates were identified by image analysis of the digital images from clones grown in the three selective media, and these data processed by the computer program as described in section 4.1.

**Example 5:** Identification of individual members of the interaction

The interaction library constructed for this example was composed of known fusion proteins with predicted interactions as shown in Figure 8. A real positive clone from this defined interaction library is therefore expected to express the interacting fusion protein-pairs LexA-SIM1 & GAL4ad-ARNT,

LexA-HD1.6 & GAL4ad-HIP1 or LexA-HD3.6 & GAL4ad-HIP1 and hence contain the corresponding pairs of plasmid constructs pBTM117c-SIM1 & pGAD427-ARNT, pBTM117c-HD1.6 & pGAD427-HIP1 or pBTM117c-HD3.6 & pGAD427-HIP1, respectively. The identification of individual members that comprise an interaction between fusion proteins that are expressed within a single cell can be made by a variety of means as outlined in Figure 1, Figure 6 and Figure 7. Three independent methods, nucleic acid hybridisation, PCR and DNA sequencing were used to identify the individual plasmid constructs that expressed the interacting fusion proteins in the positive clones 06L22 and 08N24.

#### 5.1 Identification of individual members of the interaction by nucleic acid hybridisation

The four membranes which had been placed on the SD-leu-trp-his medium and had not been used to assay  $\beta$ -gal activity were processed according to the procedure described in Larin & Lehrach (1990) in order to affix the DNA contained within the clones of the interaction library onto the surface of the membrane. A 1.1 kb DNA fragment of SIM1 and a 1.3 kb DNA fragment of ARNT were radioactively labeled by standard random priming procedures for use as a hybridisation probe (Feinberg & Vogelstein, 1983). Each probe was heat denatured for 10 min at 95 °C and hybridised overnight at 65 °C in 15 ml of 5% SDS/0.5M sodium phosphate (pH 7.2)/1 mM EDTA with a high-density spotted membrane with DNA from the interaction library affixed to it as prepared above. The membranes were washed once in 40mM sodium phosphate/0.1%SDS for 20 min at room temperature and once for 20 min at 65 °C before wrapping each membrane in Saran wrap and exposing it overnight to a phosphor-storage screens (Molecular Dynamics, USA). A digital image of each hybridised membrane was obtained by scanning the phosphor-storage screen using a phosphor-imager (Molecular Dynamics, USA). The digital image was stored on computer and was analyzed using the image analysis system for the analysis

of DNA arrays as described in Lehrach et al., 1997 which marked positive hybridisation signals with square blocks. Figure 13 shows a magnified region of each hybridised membrane corresponding to that shown in Figure 11a containing the clones 06L22 and 08N24, the spotting position of which are circled. These clones were predicted to express either the interacting fusion protein-pairs LexA-SIM1 & GAL4adARNT, LexA-HD1.6 & GAL4ad-HIP1 or LexA-HD3.6 & GAL4ad-HIP1, and hybridisation with the specific SIM1 and ARNT probes have shown that both clones contain the plasmid constructs pBTM117c-SIM1 and pGAD427-ARNT.

## 5.2 Identification of the individual members of the interaction by nucleic acid amplification and sequencing

The individual clone 06L22 was recovered from the frozen plates of the original interaction library and inoculated into SD-leu-trp-his liquid medium. This culture was allowed to grow for 3 days at 30 °C and the corresponding plasmids contained in the clone were isolated using a QiaPrep (Qiagen, Hilden) procedure. Duplex PCR was used to simultaneously amplify the inserts contained within the plasmid constructs using primer-pairs specific for either the pBTM117 or pGAD427 plasmids. The presence of the SIM1 and ARNT inserts was confirmed for clone 06L22 by electrophoresis of the amplified PCR products against separate control amplifications of the inserts from plasmids pBTM117c-SIM1 and pGAD427-ARNT as size markers (Figure 14).

PCR of the individual inserts from individual plasmids carried by clone 06L22 was conducted as above except by using only the respective primer pair for the required plasmid. The individual inserts were also amplified directly from the yeast culture using a Whole Cell Yeast PCR Kit (Bio 101, USA). The pairs of inserts isolated from clone 06L22 either by amplification from the extracted plasmid DNA or by direct PCR of the yeast clone were subjected to DNA sequencing by standard protocols.

The 1.26Kb inserts amplified using the primers specific to plasmid pBTM117 were confirmed as the expected fragment of the SIM1 gene by comparison of the known sequence for this gene (Probst et al., 1997). Likewise, the 1.37Kb inserts amplified using the primers specific to the pGAD427 plasmid were confirmed as the expected fragment of the ARNT gene.

**Example 6:** Detection and identification of interacting proteins using a large-scale and automated application of the improved 2-hybrid system

A scheme utilizing the method of the invention within a large-scale and automated approach for the parallel detection of clones that express interacting fusion proteins and the identification of members comprising the interactions is shown in Figure 6. Yeast clones from an 'interaction library' that express interacting proteins are identified on a large-scale by the use of visual inspection or digital image processing and analysis of high-density gridded membranes on which their  $\beta$ -galactosidase activity has been assayed after growth on various selective media. Automated methods as described in earlier examples are used to effect the production of the interaction library and high-density spotted membranes, and the analysis of digital images of the  $\beta$ -gal assay and hybridisation images.

#### 6.1 Generation of an interaction library for a higher Eukaryote

A random-primed and size selected (1-1.5Kb) cDNA library of 40-hour post fertilisation Sea Urchin embryos (*Strongylocentrotus purpuratus*) cloned into the *Not* 1/*Sal* 1 sites of pSport1 by standard procedures (Life Technologies, USA) was obtained as a gift from A.Poustka. 100 ng of this library, representing the estimated 6000 different transcripts expressed at this developmental stage (Davidson, 1986), was transformed into electro-competent *E.coli* cells by standard

electroporation techniques. Recombinant clones were selected by plating the transformation mixture on 2xYT/100 µg/ml ampicillin contained in 24 x 24 cm agar-trays (Genetix, UK). After growth for 18 hours at 37 °C, the resulting recombinant colonies (estimated to be 20,000 per tray) were washed from the 5 trays using 50 ml of LB liquid media for each tray. The amplified cDNA library cloned into pSport was isolated from this wash mixture by a QiaPrep (Qiagen, Germany) plasmid extraction procedure. Approximately 1 µg of the library inserts were then isolated from the plasmid DNA by Not 1/Sal 1 digestion and size selected (1 ⌘ 1.5Kb) by agarose gel purification using standard procedures.

Two pools representing all three reading frames of the two vector series pGAD428 and pBTM118 were prepared by Not 1/Sal 1 digestion and pooling of 1 µg each of vectors pGAD428 a, b & c and pBTM118 a, b & c respectively. The insert mixture that was isolated as above was split into two equal fractions and 300 ng was ligated with 50 ng of each prepared vector-series pool. Following ligation, each reaction was then separately transformed into electro-competent *E.coli* cells, and recombinant clones for each library were selected on five 24 x 24 cm plates using kanamycin or ampicillin for the pGAD428 or pBTM118 libraries respectively. Approximately 500 µg of the pBTM118 and 500 µg of the pGAD428 libraries was extracted from the two sets of *E.coli* transformants by washing off the plated cells and a subsequent QiaPrep plasmid extraction of the wash mixture as described above.

To generate the interaction library, molar-equivalent amounts of the DNA binding and activation domain libraries were pooled, and 20 µg of this mixture was co-transformed into the yeast strain L40cc by the method of Gietz et al. (1992). The resulting transformation mix was plated on a single 24 x 24 cm agar tray. The agar-trays were prepared as described in section 1.3.1. A total of twenty transformations were prepared and plated onto separate agar trays yielding an average of

1500 yeast colonies per tray after 7 days of incubation at 30°C.

#### 6.2 Creation of a regular grid-pattern of an interaction library in microtiter plates

To create a regular grid-pattern of the interaction library, the agar-trays containing yeast colonies were placed in the modified laboratory picking robot and individual clones were automatically picked as described in section 3.1. A total of 30 384-well microtiter plates were generated and represented an interaction library of greater than 10,000 clones for the study organism. After growth of yeast clones in the wells of the microtiter plate, the library was replicated to generate 3 further copies, labelled and all copies were stored at -70°C to provide for analysis at a later date as described in section 3.1.

#### 6.3 Creation of a regular grid-pattern of an interaction library on planar carriers

To provide for efficient analysis of the interaction library, the clones comprising it were arrayed at high density on 222 x 222 mm porous membranes (Hybond N+, Amersham, UK) using the method described in section 3.3. A total of twenty replica membranes, each arrayed in a '3 x 3 duplicate' regular grid-pattern of clones using 23 384-well microtiter plates from a thawed copy of the stored interaction library were produced. On each replica membrane, one microtiter plate was additionally arrayed in position 24 containing 8 different control clones representing known positive, negative and false positive clones.. This pattern corresponded to over 9000 yeast two-hybrid clones spotted at a density of approximately 40 clones cm<sup>-2</sup>. To ensure the number of yeast cells on each spot was sufficient for the four membranes which were to be placed on the counterselection media plates, the robot was programmed to spot onto each spot position 5 times from a slightly different

position within the wells of the microtiter plates. The robot created a data-file in which the spotting pattern produced and the barcode that had been automatically read from each microtiter plate was recorded.

Each membrane was carefully laid onto approximately 300 ml of solid agar media in 24 x 24 cm agar-trays. Fourteen membranes were transferred to SD-leu-trp-his media and three each of the membranes which had been spotted five times were transferred to either SD-trp+CHX or SD-leu+CAN media. The yeast colonies were allowed to grow on the surface of the membrane by incubation at 30 °C for 3 days.

#### 6.4 Detection of the readout system in a regular grid pattern and analysis using digital image analysis to identify positive clones

To provide for the efficient identification of individual clones that expressed interacting fusion proteins, the activation state of the individual clones grown on the porous carriers was examined in a highly parallel manner. The replica arrays of the interaction library grown on the six membranes placed on the counterselective media, plus three further membranes which were placed on SD-leu-trp-his medium as described above, were assayed for lacZ activity, a digital image of each was captured and image-processed as described in section 1.4.1. Figure 15 shows an grey-scale image of readout system activation for individual clones from the interaction library arrayed in a regular grid-pattern on a membrane filter and grown on SD-leu-trp-his medium.

The activation state of the readout system for each individual clone in the regular grid-pattern grown on the three selective media was recorded from each digital image using the image analysis system described in section 4.1. These data were collected for the interaction library grown on three replica-membranes for each of the selective media SD-leu-trp-his, SD-



leu+CAN & SD-trp+CHX, and was related together for each individual clone using the computer program shown in Figure 12a.

This program was used to query these data and identify those clones that had activated the readout system when grown on two out of three SD-leu-trp-his replica membranes, but not when grown on any of the two sets of three replica membranes placed on the two counterselective media SD-leu+CAN or SD-trp+CHX. The data-base correctly identified the eight different control clones each arrayed in 48 wells of the 24<sup>th</sup> microtiter plate. A total of 7539 clones from the interaction library arrayed in 23 384-well microtiter plates were thus identified as positive clones - clones that only activated the readout system when both plasmids (and hence fusion proteins) were expressed in the cell. 3983 clones were identified as false-positive clones as they also activated the readout system when grown on SD-trp+CHX medium  $\otimes$  the growth medium that eliminated the plasmid expressing the activation domain fusion protein. 113 clones were identified as false positive clones by activating the readout system when grown on SD-leu+CAN medium  $\otimes$  the growth medium that eliminated the plasmid expressing the DNA binding fusion protein. These data were automatically made available to a table of the relational database holding information on each clone of the interaction library as described in Example 7.

This relatively high number of false-positive clones identified following SD-trp+CHX selection can be explained since on elimination of the activation domain plasmid, the DNA-binding domain fusion protein is tested for its ability to activate the readout system without any partner protein. It is known that many transcripts expressed in early Sea Urchin embryos are transcription factors, and that fragments of transcription factors can commonly cause false positives in the yeast two-hybrid system when expressed as the DNA-binding domain fusion protein. Therefore, these results demonstrate

that the above method can efficiently eliminate large-numbers of false positive clones from a large-scale library vs. library screen interaction screen.

#### 6.5 Identification of the individual members of the interaction by nucleic acid amplification and sequencing

A total of 96 positive clones were randomly selected from the database and recovered from a frozen copy of the interaction library clones stored in 384-well microtiter plates. The DNA sequences cloned into the pGAD428 and pBTM118 vectors carried by each clone were directly amplified as described in section 5.2 except that the direct PCR reactions were conducted in 96-well microtiter plates using a high-throughput water-bath thermocycling machine (Maier et al., 1994).

Standard sequencing approaches were employed to characterise the nucleic acids encoding the DNA-binding domain fusion proteins of the positive clones following pBTM428- specific 96-well PCR as described above. Similarly, the sequence of the insert encoding for the activation-domain fusion protein following pGAD118-specific PCR was determined. Sequence comparison of these insets against published DNA sequences using standard sequence comparison tools (e.g. BAST), identified that one interaction involved two previously unidentified gene fragments that were expressed by the positive-clone located in plate 5, well K20. From the predicted protein sequence these two genes were designated Protein A and Protein B.

#### 6.6 Identification of individual members of the interaction by nucleic acid hybridisation

Regular grid patterns of the nucleic acids encoding the fusion proteins from the interaction library were constructed. The membranes which had been placed on the SD-leu-trp-his medium and had not been used to assay  $\beta$ -gal activity were processed according to the procedure described in Larin & Lehrach (1990)

in order to affix the DNA contained within the clones of the interaction library onto the surface of the membrane. The DNA fragment that encoded Protein A isolated as above, was radioactively labelled by the method of Feinberg & Vogelstein (1983). This labelled probe was hybridised to an array with DNA from the interaction library affixed to it, and the array washed and detected as 5.1.

The number and identity of hybridisation-positive clones was determined for each hybridisation using the automated image analysis system described in Lehrach et al., (1997). Seven clones from the interaction library were identified as hybridisation-positive for the probe encoding Protein A. Figure 16 shows a digital image of a DNA array hybridised with the gene fragment encoding Protein A with the hybridisation-positive clones identified and marked by the automated image analysis system, and Figure 17 represents a graphical representation of the positives found by this analysis. The database described in Example 7 was used to refer to the list of clones generated by the image analysis program and identify those hybridisation-positive clones that were interaction-positive clones and hence eliminate any false positive clones from further analysis. As expected, a hybridisation-positive clone was the clone 5K20 from which the probe corresponding to Protein A was obtained.

To extend the interaction pathway from Protein A, a second filter was hybridised with a radioactive labelled probe generated from the fragment coding for Protein B. Analysis of the hybridisation signals with the database described in Example 7 resulted in the identification of eight interaction-positive clones that carried the gene fragment encoding for Protein B. Figure 18 shows a graphical representation of the hybridisation-positive and interaction-positive clones identified with probe B (open circles) and probe A (red circles). Two clones (5K20 and 3L11 marked by "A/B") gave a hybridisation signal with both probe A and Probe B, indicating

that both these positive clones expressed the same interacting fusion proteins.

To further extend the interaction pathways of proteins A and B, the DNA binding and activation domain plasmids were extracted from one interaction-positive clone that gave a hybridisation signal only with probe B (clone 6D18). DNA sequencing of the inserts carried by these genetic elements confirmed the presence of a gene fragment encoding for Protein B in the DNA binding domain plasmid. Sequence analysis showed that the activation domain plasmid carried a fragment for another unknown gene coding for Protein C. This gene fragment was used as a probe to another array and the data analysed as above. Figure 19 shows the results of this hybridisation (marked with diamonds), together with that from the previous two hybridisations. A total of six interaction-positive clones were identified as carrying genetic elements encoding for Protein C. Three of these interaction-positive clones were previously shown to hybridise with probe B (4G19; 1D7; 6D18) and two clones to hybridise with probe A (1C22; 3A11). A graphical view of the interactions identified by these three simple hybridisations is outlined in Figure 19. Question marks represent possible further steps in the network which could be further investigated by a similar investigation of the genetic elements carried by the remaining hybridisation-positive clones for probes A, B or C. Indeed, by following this focused hybridisation approach, 14 different protein-protein interactions were identified by a total of nine hybridisations and subsequent sequencing of the inserts encoding the interacting members. All these data were entered into the database described in Example 7.

#### 6.7 Automated rearraying of positive clones

The 3443 positive clones identified as described above were distributed across all 23 microtiter plates of the interaction library. To greatly facilitate further analysis of positive

clones, it was advantageous to individually physically isolate clones and to generate a second, re-arrayed regular grid-pattern of positive clones, preferably within a further set of 384-well plates.

Existing rearranging robots such as described by Stanton et al, (1996), Lehrach et al., (1997) or those sold by commercial sources (Genetix, UK) system failed to provide a satisfactory inoculate when transferring yeast cells from individual wells of a source ('mother') 384-well plate containing the original interaction library in wells of a new, sterile 384-well destination ('daughter') plate containing growth medium. Therefore, the existing transfer pins were replaced by straight 2 mm diameter pins that terminated in a flat end. Secondly, the inoculation procedure was modified to maximise the amount of dried cell material carried on the pin that was transferred into the new well within the daughter plate as described for automated picking of yeast colonies in section 3.1. The pins were sterilised between rearranging cycles by a 0.3% hydrogen peroxide wash-bath, 70% ethanol wash-bath and heat-drying procedure as described in section 3.1.

The list of positive clones, together with their plate-well location was generated from the data-base described in Example 7 and automatically loaded as a computer file onto the rearranging robot. The robot automatically took the mother plate containing the first positive yeast two-hybrid clone by reference to the data file and read and recorded the barcode of the plate. Individual and sequential pins of the 96-pin rearranging head were positioned above and lowered into the required wells from this first plate, and the mother plate was automatically exchanged when all positive clones had been sampled. When all 96-pins had been used to collect inoculates of positive clones, the head was automatically moved over to the first 384-well daughter plate containing SD-leu-trp/7%glycerol and inoculated all 96-pins in the first set of wells as described above. A data output file was then updated

which related the new plate-well location of a given positive clone in the re-arrayed library to its old plate-well location in the original interaction library. All pins were then sterilised as described, and the cycle completed until all positives clones had been transferred from the interaction library to a new plate-well location comprising the re-arrayed library. The data output file was then transferred to the central computer database to append a table in the data-base described in Example 7 to record the correct location of a given positive clone in the re-arrayed interaction library. The resulting clones in the daughter plates were replicated into two further copies and stored at  $\times 70^{\circ}\text{C}$  as described in section 3.1.

**Example 7: Generation of a data-base of interactions.**

Central to the scheme (Figure 2) is a data-table holding relevant information on each member of an interaction - the cDNA-Table - where a separate record in the table represents each member of an interaction, and members are indicated to form interactions by sharing the same clone name. It is advantageous to structure the core data-table in this way for several reasons. First, the same core table can be used to hold data on cDNAs from different kinds of genetic libraries (for example, standard cDNA or genomic libraries) which can be generated during a global analysis using various genomic techniques, not just interaction data. Secondly, each of the members of an interaction, or genetic fragments may be further characterised by a number of ways for different sets of data. Of direct relevance to protein-protein interaction for a given genetic fragment in the cDNA\_Table is first, the Gene\_Table, which provides a direct relationship to the fragment's DNA sequence, nucleotide homology match (for example through BLAST searching) and the corresponding gene name. Second, the Domain\_Table provides facility to directly access data of the fragment's in-frame translation, amino acid homology match (for example through BLASTN searching) and any 2 or 3-

dimensional structural information which may be known or can be predicted. As is commonly known in molecular biology, there are many ways in which a given genetic fragment may be characterised, and this data-base structure provides the facility to relate from the central cDNA\_Table to any other table holding data describing said characterisation as may be appropriate. For example, those holding data on genetic, expression, target validation, protein biochemistry or library construction information. Of particular relevance to the method of invention, is the relationship of a given cDNA fragment to a table holding information on oligofingerprinting data. Said oligofingerprinting data can be used to identify each member of an interaction in a highly parallel manner and includes fields for data such as cluster number, confidence of cluster membership and predicted gene homology for that cluster (Maire et al., 1994). Third, such a data-base structure will more easily enable tertiary or higher order interactions to be incorporated within the same data table. This is in contrast to a structure in which interactions rather than members of an interaction were the basic object or record in a data table, and for each higher order interaction a new data-table would be needed or an existing data-table modified.

In the case of a yeast two-hybrid interaction screen one related table would be the Y2H\_Table. Said table may include information for a given clone pertaining to cloning and experimental details of its creation, the tissue and library from which it was derived, its physical location to enable easy access for further studies, whether it was derived from the mating of given *Mata* and *Mata $\alpha$*  strains. Importantly, the Y2H\_Table holds information pertaining to the interaction class of the clone  $\otimes$  where said interaction class is defined as whether the clone was a positive clone, negative clone, or a false positive with respect to either the activation domain (AD) or binding domain (BD) fusion protein. The value for said interaction class is easily derived for a large number of

clones by the method of invention described in earlier examples.

To assist any focused approach to identifying members comprising the interactions, the Hyb Table is provided. This table relates for a given clone, the hybridisation intensity obtained with a given probe in a hybridisation experiment using a given high density array. Said high-density array to be related to tables holding data from the spotting robot such as the defined spotting pattern used, the method by which the array was produced and the identity of the library and clones arrayed on said array. The incorporation of these tables within a user interface will enable this embodiment of the method of invention to be easily conducted by displaying to the user the physical location of a given positive yeast two hybrid clone that hybridised to a given probe. Said two-hybrid clone can then be recovered, the members comprising the interaction isolated by PCR and sequenced. Said sequenced members of an interaction then provide data to be entered into the cDNA\_Table and other related tables on further analysis. Said member to then be used as a second hybridisation probe onto an array to identify the next step in an interacting pathway by the same procedure.

On collection of a substantial number of interacting members within the cDNA\_Table, these data can be curated by manual and/or expert systems to update a definitive data table for example the PathCode\_Table. Said definitive database to hold the highest quality information on interactions from the cDNA\_Table, where said highest quality information on interactions to be those from the cDNA Table that pass a level of 'certainty' as specified to the curator and/or expert system. To assist in the decision-making process, all relevant data especially that of the translated frame of the cDNA and corresponding protein domain is related from other tables and presented in a usable form to the curator and/or expert system. This presentation allows for easy recognition and



exclusion or correction of basic errors in the data such as poor quality sequencing, or incorrectly cloned cDNA fragments. These may include contaminating fragments which can be identified as originating from an organism which is different to that of the cDNA library.

A given cDNA is entered into the PathCode\_Table only once for each interaction in which it is found, together with a record for the corresponding interacting cDNA (or cDNAs for multimer complexes). However, where a cDNA has different interactions, for example with different proteins or where different protein domains of the cDNA interacts with different proteins, then in each case a different record for the cDNA is created. These different records are linked by a common and unique 'Interaction ID'. A given interaction is represented thus only once in the PathCode\_Table, and is related to previous tables in the data-base by the host-cell clone that represents the interaction and the ID of each cDNA in the interaction. Said host-cell that represents the interaction is selected by consideration and curation of all host-cells and the interacting fragments representing said interaction held in the cDNA\_Table.

A set of criteria can be implemented to assist in said curation and selection, and to derive a measure of confidence for the interaction. As way of example, such criteria may have decreasing information value and include: First, if a given interaction is observed in both directions of the experiment ie proteinA-AD interacting with proteinB-BD, and proteinB-BD interacting with proteinA-DB. Second, if different examples of the same interaction are observed. Where different examples of the same interaction are defined as protein fragments of substantially different length and position (for example greater than 10% different) but from the same underlying protein domain and are also found to interact. Third, if the same examples of the same interaction are observed, for example by multiple cloning of the same fragments where the

same fragments are of substantially the same length and position from the same underlying protein domain. Fourth, that the protein domains that interact may have biological relevance. That is, similar domains or genes are known to interact from public literature, or it is known that both genes are expressed or likely to be expressed in the same cellular location. This criterion can also be used as an internal quality control of the library cloning, interaction experiment and subsequent identification of interacting members since every interaction experiment should identify a certain set of published 'house-keeping interactions', and the identification of such interactions can be used as quality measure for the overall interaction experiment.

One criterion of particular importance, is the optional validation of a given interaction by secondary experiments. For example, cDNA fragments representing the interacting proteins may be subcloned, and additional interaction experiments be conducted. Said additional interaction experiments may include testing each protein for interaction against a set of unrelated proteins to investigate the specificity of said interaction. Said testing may be conducted using the same interaction method that identified the interaction, for example the yeast two-hybrid, but preferable it is an independent method. Favoured, is where a given interaction is biochemically validated using methods including tissue co-northern, cellular co-localisation or co-precipitation studies.

All these criteria are considered by the curator and/or expert system to assist in the decision on which cDNA fragments and their interactions are entered into the PathCode\_Table. Other interactions known or published in scientific literature may also be entered into this data-base during the curation procedure, and hence a field in the table represents the source of this interaction being internal or an external reference. The PathCode table has relational links to

secondary or external data-bases holding data on nucleotide and protein sequences, and biochemical, structural, biological or bibliographical information. These data, representing the complete relationships between all tables and data-bases can be queried by using simple user interfaces, designed for example using Java, or by more complicated commands such as those provided by SQL. Possible queries include those to locate from these data interactions, pathways or networks for a given nucleotide or amino acid sequence or motif, or for a given 3-dimensional structure or motif. Secondly, for highly established networks, these data may be queried to identify a given pathway between two given points. It may be that some queries are more efficiently conducted using a substantially different design of the PathCode\_Table  $\otimes$  for example by representing a given interaction as the underlying record rather than a given member of an interaction. A person skilled in the art would be able to transfer data from one table design to another using standard data-parsing systems to enable said more efficient conduction of queries.

The result of these queries is displayed using graphical methods to enable the investigator to interpret these data most efficiently. Said graphical methods to include elements activated by mouse clicks such as hotlinks to seamlessly link these data with other data sources, or to query and display further levels of interactions. Computer-based methods of generating visual representations of specific interactions, partial or complete protein-protein interaction networks can be employed to automatically calculate and display the required interactions most efficiently. Both finding the network paths and calculating the optimal display of the found paths can be based on algorithms well known in the art of mathematical graph theory. For example, algorithms similar to those which have been employed to display other biological relationships such as genetic pedigrees and phylogenetic relationships.

An established computer data-base of protein interactions has many useful applications. For example, it may be used to predict the existence of new biological interactions or pathways, or to determine links between biological networks. Furthermore with this method, the function and localisation of previously unknown proteins can be predicted by determining their interaction partners. It also can be used to predict the response of a cell to changes in the expression of particular members of the networks without making a molecular, cellular or animal experiment. Finally, these data can be used to identify proteins or interactions between proteins within a medically relevant pathway, which are suitable for therapeutic intervention, diagnosis or the treatment of a disease.

**Example 8:**       Preselection against false positive clones and the automated creation of a regular grid-pattern of yeast cells expressing a fusion protein

#### 8.1 Genetic pre-selection of false positive clones

Three mating type- $\alpha$  yeast strains were constructed by co-transformation using the method of Schiestel & Gietz (1989) into L40ccu, of the plasmid pLUA containing the URA3 readout system, and either the pBTM117c, pBTM117c-SIM1 or pBTM117c-HIP1 plasmids respectively. Transformants that contained both the pLUA plasmid and one of the DNA binding domain plasmid were selected on SD-trp-ade medium. Three mating type- $\alpha$  yeast strains were similarly constructed by cotransformation into L40ccu $\alpha$  of pLUA, and either the pGAD427, pGAD427-ARNT or pGAD427-LexA plasmids respectively. Transformants that contained both the pLUA and one of the activation domain plasmids were selected on SD-leu-ade medium. The yeast strains thus obtained are listed in Table 3.

The yeast strains x1a, x2a and x3a were replica plated onto the selective media SD-trp-ade, SD-trp-ade containing 0.2% 5-FOA and SD-trp-ade-ura, while the yeast strains y1 $\alpha$ , y2 $\alpha$  and

y3 $\alpha$  were replica plated onto the selective media SD-leu-ade, SD-leu-ade containing 0.2% 5-FOA and SD-leu-ade-ura. Table 4 shows that the two yeast strains x3a and y3 $\alpha$  which expressed the fusion proteins LexA-HIP1 and GAL4ad-LexA respectively were unable to grow on their respective media containing 5-FOA yet were able to grow on their respective media lacking uracil. In contrast, all other yeast strains that contained plasmids that expressed fusion proteins that were alone unable to activate the readout system could grow on their respective media containing 5-FOA, but could not grow on selective media lacking uracil. This indicates that it is possible to eliminate yeast clones that express single fusion proteins which auto-activate the readout system, by selection on media containing 5-FOA. Thus, the *URA3* readout system successfully eliminated clones containing auto-activating fusion proteins prior to interaction mating.

## 8.2 Creation of a regular grid pattern of genetically pre-selected yeast cells expressing a fusion protein

Two defined libraries of clones that express fusion proteins were created. First, the yeast strain L40ccu was transformed with the plasmid pLUA and a resulting stable transformant colony cultured in minimal medium lacking adenine. Cells from this culture were rendered competent and transformed with 3  $\mu$ g pooled mixture of all six pBTM117c constructs shown in Table 2. Second, the yeast strain L40ccu $\alpha$  was transformed with the plasmid pLUA and a resulting stable transformant colony cultured in minimal medium lacking adenine. Cells from this culture were rendered competent and transformed with 3  $\mu$ g pooled mixture of all six pGAD427 constructs shown in Table 2. In all cases, competent cells were prepared and transformations conducted using the method of Schiestel & Gietz (1989).

The two transformation mixes were incubated at 30°C for 2 hours in 10 ml of YPD liquid medium before plating onto large

24 x 24 cm agar trays (Genetix, UK). The Mata cells containing the pBTM117c fusion library were plated onto minimal medium lacking tryptophan and adenine but containing 0.2% 5-FOA (SD-trp-ade+FOA), while the Mata $\alpha$  cells containing the pGAD427 fusion library were plated onto minimal medium lacking leucine and adenine but containing 0.2% 5-FOA (SD-leu-ade+FOA). The agar trays were poured using an agar-autoclave and pump (Integra, Switzerland) to minimise tray-to-tray variation in agar colour and depth. After plating, the colonies were grown by incubating the trays at 30°C for 4 to 7 days resulting in approximately 1500 colonies per tray.

Mata clones containing the plasmid pBTM117c-HIP1 and Mata strains containing the plasmid pGAD427-LexA expressed the fusion proteins LexA-HIP1 and GAL4ad-LexA respectively. These fusion proteins were shown to activate the URA3 readout system without any interacting fusion protein. Therefore, cells carrying these plasmids should be unable to grow on selective media containing 5-FOA. Hence, only those yeast clones expressing a single fusion protein unable to activate the URA3 reporter gene will form colonies on be picked by the modified robotic system.

Using the modified laboratory picking robot, individual yeast colonies were automatically picked from the agar-trays into individual wells of a sterile 384-well microtiter plates, as described in section 1.3.1 except that the Mata yeast strains were picked into microtiter plates containing the growth medium SD-trp-ade and 7% (v/v) glycerol, while the Mata $\alpha$  yeast strains were picked into microtiter plates containing the growth medium SD-leu-ade and 7% (v/v) glycerol. The resulting microtiter plates were incubated at 30°C for 4 days with a cell-dispersal step after 36 hours section 3.1. After incubation, each plate was replicated to create two additional copies into labelled 384-well microtiter plates and pre-filled with the liquid growth medium containing 7% glycerol as was appropriate for the yeast strain. The replicated plates were incubated at 30 °C for 4 days with a cell dispersion step

conducted after 36 hours as above, subsequently frozen and stored at -70°C together with the original picked microtiter plates of the libraries of cells expressing fusion proteins.

It will be clear that higher density regular grid-patterns of such an interaction library can be easily generated by a person skilled in the art from these microtiter plates of diploid yeast cells by following the methods disclosed in sections 3.2, 3.3 and 3.4 of this invention.

### 8.3 Visual differentiation against false positives for an improved yeast two-hybrid system

Six yeast strains were generated by transforming each of the pBTM117c plasmid constructs described in Table 2 into L40ccu by the method of Schiestel & Gietz (1989). Each strain was plated on selective growth medium lacking tryptophan, buffered to pH 7.0 with potassium phosphate and containing 2 ug/ml of the  $\beta$ -galactosidase substrate X-Gal (SD-trp/XGAL). Six further strains were similarly constructed by transforming each of the pGAD427 plasmid constructs described in Table 2 into L40ccu $\alpha$ . These strains were plated on selective growth medium lacking leucine, buffered to pH 7.0 with potassium phosphate and containing 2 ug/ml of X-Gal (SD-leu/XGAL). After incubation at 30 °C for 7 days, the strains were inspected for growth and blue colour. Table 5 shows that although all yeast strains were able to grow on the selective media, only the L40ccu strain expressing the fusion protein LexA-HIP1 and the L40ccu $\alpha$  strain expressing the fusion protein GAL4ad-LexA turned blue. In contrast, all other yeast strains that contained plasmids that expressed fusion proteins unable to activate the readout system alone could grow on the selective media, but did not turn blue. It was found that for the fusion proteins described here, the blue-colour generated by auto-activation of the  $\beta$ -galactosidase readout system developed faster than any pink-colour of other clones due to the *ade2* mutation. However, the blue colour may develop slower than the pink colour for some

fusion proteins that may affect the reliability of visual differentiation using automated systems with grey-scale vision systems. Therefore, a person skilled in the art will be able to incorporate colour recognition systems, colour filters or construct a yeast strain that does not develop the pink colour. For example, using a strain carrying the wild-type ADE2 gene, or the complementary mutation *ade3*.

#### 8.4 Using automation to visually discriminate false-positive yeast clones and the creation of a regular grid pattern of cells

Two defined fusion protein libraries were generated. Six pBTM117c constructs shown in Table 2 were pooled and 3  $\mu$ g of the mixture was co-transformed into the yeast strain L40ccu. The resulting transformants were selected by plating the mixture onto five large 24 x 24 cm agar-tray (Genetix, UK) containing minimal medium lacking tryptophan, buffered to pH 7.0 with potassium phosphate and containing 2  $\mu$ g/ml of X-Gal (SD-trp/XGAL). Second, the six pGAD427 constructs shown in Table 5 were pooled and 3  $\mu$ g of the mixture was co-transformed into the yeast strain L40ccu $\alpha$ . The resulting transformants were selected by plating the mixture onto five large 24 x 24 cm agar-tray (Genetix, UK) containing minimal medium lacking leucine, buffered to pH 7.0 with potassium phosphate and containing 2  $\mu$ g/ml of X-Gal (SD-leu/XGAL). These agar-trays were poured using an agar-autoclave and pump (Integra, Switzerland) to minimise tray-to-tray variation in agar colour and depth. The agar-trays were incubated for 7 days to allow the yeast clones to grow and the blue colour of clones able to activate the  $\beta$ -galactosidase reporter gene to develop. In all cases, competent cells were prepared and transformations conducted using the method of Schiestel & Gietz (1989).

Using the modified laboratory picking robot, individual yeast colonies were automatically picked from the agar-trays into individual wells of a sterile 384-well microtiter plates, as



described in section 3.1 except that the *Mata* yeast strains were picked into microtiter plates containing the growth medium SD-trp and 7% (v/v) glycerol, while the *Mata $\alpha$*  yeast strains were picked into microtiter plates containing the growth medium SD-leu and 7% (v/v) glycerol

Automated visual differentiation was made by using the blue-white sorting parameters described in section 3.1. The robot was programmed to pick only white colonies into microtiter plates and ignore all colonies that had turned blue on activation of the  $\beta$ -galactosidase reporter gene. Figure 20 displays automated visual discrimination of false positive clones using the modified picking system described above. The resulting microtiter plates were incubated at 30°C for 4 days with a cell-dispersal step after 36 hours section 3.1. After incubation, each plate was replicated to create two additional copies into labelled 384-well microtiter plates and pre-filled with the liquid growth medium containing 7% glycerol as was appropriate for the yeast strain. The replicated plates were incubated at 30 °C for 4 days with a cell dispersion step conducted after 36 hours as above, subsequently frozen and stored at -70°C together with the original picked microtiter plates of the libraries of cells expressing fusion proteins.

It will be clear that higher density regular grid-patterns of such an interaction library can be easily generated by a person skilled in the art from these microtiter plates of diploid yeast cells by following the methods disclosed in sections 3.2, 3.3 and 3.4 of this invention.

Only those colonies that expressed the fusion protein LexA-HIP1 or the GAL4ad-LexA should be able to activate the LacZ gene and hence turn blue when grown on the selective medium. Therefore, blue colonies from the *Mata* library would be expected to carry the pBTM117c-HIP1 construct while white colonies would carry other pBTM117c plasmid constructs. Likewise, blue colonies from the *Mata $\alpha$*  library would be

expected to carry the pGAD427-LexA construct while white colonies would carry other pGAD427 plasmid constructs. To prove this hypothesis, 10 white and 10 blue colonies were randomly selected from a picked agar-tray of the *Mata* library, and twenty colonies from a 384-well microtiter plate that had been automatically picked from this plate. All 40 colonies were hand inoculated into individual 1ml liquid cultures of SD-trp medium and the cultures grown for 3 days at 30°C. The insert carried by each clone was checked by direct PCR amplification of the pBTM117c insert from the yeast culture and DNA sequencing by standard protocols. All ten yeast colonies that had activated the readout system and turned blue carried the 1.2 Kb HIP1 fragment, while the white colonies carried the 1.6 Kb HD1.6, the 1.1 Kb SIM insert or gave no amplification reaction from the non-recombinant vector. Of the twenty clones selected from the 384-well microtiter plate which had been automatically visually differentiated, none carried the 1.2 Kb HIP1 fragment. A similar experiment of clones manually selected and automatically picked from the *Mata* library confirmed that blue colonies contained the LexA insert from the pGAD427-LexA construct, and no automatically picked colonies carried this insert. The pBTM117c-HIP1 plasmid encoded for the LexA-HIP1 fusion protein, and the pGAD427-LexA encoded for the GAL4ad-lexA fusion protein were known to auto-activate the readout system without any partner protein. Hence, automatic visual differentiation has preselected against these false positive clones and automatically created a regular grid pattern of yeast clones expressing a single fusion protein unable to activate the readout system.

**Example 9:** Automated interaction mating to combine genetic elements in yeast cells

9.1 Automated interaction mating on a solid support in regular pattern

The yeast strains that did not express auto-activating fusion proteins in section 8.1 were mated using an automated approach. Each of the yeast strains x1a, x2a, y1 $\alpha$  and y2 $\alpha$  was grown in every well of one of four microtiter plates containing SD-trp-ade medium for the Mata strains and SD-leu-ade medium for the Mata $\alpha$  strains. Each plate was labelled with a unique barcode and using a spotting robot such as described by Lehrach et al. (1997), the yeast strains x1a and x2a were transferred in a defined 2 x 2 duplicate pattern with an inter-spot spacing of 2mm to Hybond-N+ membrane (Amersham) which had been pre-soaked with YPD medium. The spotting robot then automatically transferred the yeast strains y1 $\alpha$  and y2 $\alpha$  to the same respective spotting positions on each membrane as, and already containing the x1a and x2a clones. The robot automatically sterilised the spotting tool, changed the microtiter plate between each set of clones transferred and created a data-file in which the spotting pattern produced and the barcode that had been automatically read from each microtiter plate was recorded. The spotted membranes were transferred to YPD plates and incubated for over night at 30°C to allow mating and growth to occur. Each membrane was assayed for  $\beta$ -Gal activity using the method of Breeden & Nasmyth (1985) and was subsequently air dried overnight. A digital image of each dried filter was captured using a standard A3 computer scanner and image processed as described in section 4.1. The processed image was stored on computer and the identity of clones that expressed  $\beta$ -Galactosidase was determined using the image analysis system described in section 4.1. Figure 21 shows the results of automated interaction mating between the strains x1a & y1 $\alpha$  and x2a & y2 $\alpha$ . Both resulting diploid strains grew on YPD media, yet only the diploid strain resulting from the interaction mating of x2a & y2 $\alpha$  that contained plasmids encoding the interacting fusion proteins LexA-SIM1 & GAL4ad-ARNT respectively, showed a LacZ+ phenotype and turned blue on incubation with X-Gal. No  $\beta$ -galactosidase activity was observed for the diploid strain resulting from the interaction mating between the strains x1a

and y1 $\alpha$  that contained plasmids encoding the proteins LexA and GAL4ad.

## 9.2 Automated interaction mating based on liquid culture

Two defined libraries of clones which express fusion proteins were created. First, the yeast strain L40ccu was transformed with the plasmid pLUA and a resulting stable transformant colony cultured in minimal medium lacking adenine. Cells from this culture were rendered competent and transformed with 3  $\mu$ g pooled mixture of all six pBTM117c constructs shown in Table 2. Second, the yeast strain L40ccu $\alpha$  was transformed with the plasmid pLUA and a resulting stable transformant colony cultured in minimal medium lacking adenine. Cells from this culture were rendered competent and transformed with 3  $\mu$ g pooled mixture of all six pGAD427 constructs shown in Table 2. In all cases, competent cells were prepared and transformations conducted using the method of Schiestel & Gietz (1989).

The cells in the two resulting transformation mixes were allowed to recover by incubation at 30°C in YPD liquid medium for 2 hours before plating onto large 24 x 24 cm agar trays (Genetix, UK). The Mata cells containing the pBTM117c fusion library were plated onto minimal medium lacking tryptophan and adenine but containing 0.2% 5-FOA (SD-trp-ade+FOA), while the Mata $\alpha$  cells containing the pGAD427 fusion library were plated onto minimal medium lacking leucine and adenine but containing 0.2% 5-FOA (SD-leu-ade+FOA).

The colonies on the agar-trays were grown by incubation at 30°C for 4 to 7 days. To minimise false positives arising from dormant cells, the colonies on the two agar-trays were replica-plated onto new agar-trays containing the same respective selective media as a given original tray using standard velvet replication. This replication procedure only transferred cells from the top of a growing colony and thus

reduced the carry over of dormant cells and hence the number of false positive clones in the yeast two-hybrid system. These replica agar-trays were incubated at 30°C for 4 to 7 days in order for the yeast cells to grow.

To conduct the liquid interaction mating, the resulting *Mata* and *Mata* colonies were separately collected off both replica trays by washing with 20 ml of liquid minimal medium. These two mixtures of yeast clones were carefully resuspended, pelleted and washed with sterile distilled water before incubation in 100 ml of YPD in order to ensure that the cells in both mixtures were mating competent. The two populations of mating competent cells were combined in 500 ml of YPD liquid media contained within a 10 litre flat bottomed flask and incubated at 30°C with very gentle shaking (< 60 rpm) overnight to allow interaction mating to proceed. The resulting mixture of diploid cells was pelleted by gentle centrifugation at 3000 rpm for 5 min, washed twice with 50 ml of sterile distilled water and finally, 10 ml of the resulting cell suspension was plated onto each of five 24 x 24 cm agar-trays containing 300 ml of minimal medium lacking leucine, trptophan, adenine, histidine and uracil (SD-leu-trp-ade-his-ura). The agar trays were poured using an agar-autoclave and pump (Integra, Switzerland) to minimise tray-to-tray variation in agar colour and depth. After plating, the colonies were grown by incubating the trays at 30°C for 4 to 7 days.

After incubation, the resulting diploid yeast cells expressing interacting fusion proteins were automatically picked using our modified picking system as described in section 3.1 except that the picked clones were inoculated into microtiter plates containing the liquid selective medium SD-leu-trp-ade/7% glycerol. The interaction library comprising the diploid yeast cells contained in the microtiter plates were grown by incubation at 30°C as described in section 3.1. Two further copies of the interaction library were made into new microtiter plates containing SD-leu-trp-ade/7% glycerol growth

medium, all plates were individually labelled with a unique barcode and stored at  $\times 70^{\circ}\text{C}$  until required for further analysis as described in section 3.1.

It will be clear that higher density regular grid-patterns of such an interaction library can be easily generated by a person skilled in the art from these microtiter plates of diploid yeast cells by following the methods disclosed in sections 3.2, 3.3 and 3.4 of this invention. The creation of high-density regular grid patterns of diploid yeast cells can be conducted using the procedures as described in earlier sections. These arrays can be used to assay reporter gene activity, or for generation of nucleic acid arrays for hybridisation. Modifications to selective medium may be required which a person skilled in the art will recognise.

**Example 10:** Application of the improved two-hybrid system to a prokaryotic two-hybrid system

#### 10.1 Strains, readout systems and vectors

Two *E.coli* strains KS1-OR2HF<sup>+</sup> and KS1-OR2HF<sup>-</sup> were created that carry the *sacB* counterselective marker under the control of the *placO<sub>R</sub>2-62* promoter, and also the tetracycline selective gene under the control of a second *placO<sub>R</sub>2-62* promoter. Both strains have the *sacB* counterselective reporter gene stably inserted within the *E.coli* chromosome by knock-out of the arabinose operon to enable arabinose controlled inducible promoters to be utilised. The selective Tet. reporter gene is stably inserted in within the chromosome by knock-out of the lactose operon which also enables a *lacY* counterselective marker to be utilised. Strain KS1-OR2HF<sup>+</sup> was created by transformation of the fertility conferring F' plasmid into KS1-OR2HF<sup>-</sup>. KS1-OR2HF<sup>-</sup> was created by site-specific knock-out and insertion of the *sacB* reporter gene construct into the arabinose operon of strain KS1-ORTet by transformation of the plasmid pK03-araO<sub>R</sub>sacB and subsequent selection for stable

insertions using the method of Link et al. (1997) pKO3-araOrsacB was prepared by blunt-ended ligation of a 1.4 Kb OrsacB fragment into *Stu* I digested pKO3-ARA to produce an insert of the OrsacB fragments flanked by 2.5 Kb bp and 1.0 Kb of the 3' and 5' ends of the *E.coli* arabinose operon respectively. pKO3-ARA carries the complete arabinose *E.coli* operon which had been amplified by PCR from *E.coli* genomic DNA using tailed primers, digested with *Sal* I and cloned into the *Sal* I site of pKO3 by standard procedures. The OrsacB fragment was created by ligating together PCR fragments of the *placO<sub>R</sub>2-62* promoter and the *sacB* gene. The *placO<sub>R</sub>2-62* promoter and *sacB* PCR fragments were amplified using standard procedures and anchor primers which gave rise to complementary overhangs between the two consecutive fragments which were subsequently annealed to generate the chimeric sequence (see, for example, Current Protocols in Molecular Biology, Eds. Ausubel et al. John Wiley & Sons: 1992) from the plasmids KJ306-31 and pKO3. The *lac* promoter derivative *placO<sub>R</sub>2-62* carried by the plasmid KJ306-31 was prepared by cleaving the plasmid KJ306 with *Hinc* II and inserting a 31bp linker sequence (Dove et al. 1997). The strain KS1-ORTet was created by site-specific knock-out and insertion of a tetracycline reporter gene under the control of the *placO<sub>R</sub>2-62* promoter into the lactose operon of strain KS1F<sup>-</sup> also by genomic knock-out utilising the pKO3 system. The tetracycline gene was obtained by PCR of the plasmid pACYC184. Modifications to the above knock-out insertion method were made to make an appropriate pKO3 construct to enable the knock-out insertion of the chimeric tetracycline reporter gene into the lactose operon as will be possible by a person skilled in the art. The *E.coli* strain KS1F<sup>-</sup> was constructed from KS1 (Dove et al.) by removal of the F' plasmid using standard plasmid curing procedures.

Two vectors, pBAD18- $\alpha$ RNAP and pBAD30-cI were constructed to provide further genetic features to enable the method of invention (Figure 22). The vectors are based on the pBAD series of vectors which provide tight inductive control

expression of cloned genes using the promoter from the arabinose operon (Guzman et al., 1995 J. Bact. 177: 4141-4130, and can be maintained in the same *E.coli* cell by virtue of compatible origins of replication. The plasmid pBAD18- $\alpha$ RNAP expresses under the control of the arabinose promoter, fusion proteins between the  $\alpha$  amino terminal domain (NTD) of the  $\alpha$ -subunit of RNA polymerase and DNA fragments cloned into the multiple cloning site. The presence of this plasmid in kanamycin sensitive cells can be selected by plating on growth medium supplemented with kanamycin, or for its absence by the counterselective *rpsL* allele by plating on media supplemented with streptomycin (Murphy et al. 1995). The plasmid pBAD30-cI expresses under the control of the arabinose promoter, fusion proteins between the  $\lambda$ cI protein and DNA fragments cloned into the multiple cloning site. The presence of this plasmid in ampicillin sensitive cells can be selected by plating on growth medium supplemented with ampicillin, or for its absence by the counterselective *lacY* gene by plating on media supplemented with 2-nitrophenyl- $\beta$ -D-thiogalactosidase (tONPG) (Murphy et al. 1995). Additionally, the 288 bp oriT sequence enables unidirectional genetic exchange of the pBAD30-cI plasmid and its derivatives from *E.coli* cells containing the F' fertility factor to F<sup>-</sup> strains lacking the fertility factor.

The plasmid pBAD18- $\alpha$ RNAP was constructed by cloning a 0.7 Kb DNA fragment encoding the  $\alpha$  amino terminal domain (NTD) (residues 1-248) of the  $\alpha$ -subunit of RNA polymerase ( $\alpha$ -NTD) into *Eco* RI digested pBAD18-CS. The 0.7 Kb  $\alpha$ -NTD fragment was isolated by PCR from the plasmid pHTf1 $\alpha$  (Tang et al., 1994 Genes Dev 8: 3058-3067). The plasmid pBAD18-CS was obtained by site-specific insertion assisted by PCR cloning of the 400 bp coding region and translational start site of the *rpsL* allele into pBAD18-Kan (Guzman et al 1995) before the transcriptional termination signal of the kanamycin gene to enable polycistronic transcription of the counterselective and



selective markers. The *rpsL* allele was obtained by PCR amplification of the plasmid pNO1523 (Murphy et al. 1995).

The plasmid pBAD30-cI was constructed by cloning a 730 bp DNA fragment encoding the  $\lambda$ cI protein into *Eco* RI digested pBAD30-TCS. The 730 bp fragment encoding the  $\lambda$ cI protein was isolated by PCR from the plasmid pAC $\lambda$ cI (Dove et al 1997). The plasmid pBAD30-TCS was obtained by site-specific insertion assisted by PCR cloning of the 1.3 Kb coding region and translational start site of the *lacY* gene into pBAD30-T before the transcriptional termination signal of the ampicillin gene to enable polycistronic transcription of the counterselective and selective markers. The *lacY* gene was obtained by PCR amplification of the plasmid pCM10 (Murphy et al. 1995). The plasmid pBAD30-T was obtained by site specific insertion of a 288 bp oriT sequence obtained by PCR from the F' plasmid between the M13 intergenic region and *cat'* locus of pBAD30 (Guzman et al 1995).

#### 10.2 Detection and identification of interacting proteins using a large-scale and automated prokaryotic two-hybrid system

Generation of a libraries of *E.coli* cells expressing fusion proteins

The pSport1 plasmid extraction containing the amplified cDNA library of *Strongylocentrotus purpuratus* described in section 6.1 was used. Approximately 1  $\mu$ g of the library inserts were then isolated from the plasmid DNA by *Hind* III/*Sal* I digestion and size selective (1-1.5Kb) agarose gel purification using standard procedures.

The two plasmids pBAD18- $\alpha$ RNAP and pBAD30-cI were prepared by digestion with *Hind* III/*Sal* I. The insert mixture that was isolated as above was split into two equal fractions and 300 ng was ligated with 50 ng of each of the two prepared

plasmids. Following ligation, the pBAD18- $\alpha$ RNAP reaction was then transformed into competent KS1-OR2HF<sup>-</sup> *E.coli* cells, and the pBAD30-cI was transformed into competent KS1-OR2HF<sup>+</sup> *E.coli* cells.

Genetic preselection against false positive clones and the automated creation of a regular grid-pattern of *E.coli* cells expressing a fusion protein

The two transformation mixes were plated onto large 24 x 24 cm agar trays (Genetix, UK) containing selective media. The F<sup>-</sup> cells containing the pBAD18- $\alpha$ RNAP fusion library were plated onto LB selective medium supplemented with kanamycin (50 ug/ml), arabinose (0.2% w/v) and sucrose (5% w/v). The F<sup>+</sup> cells containing the pBAD30-cI fusion library were plated LB selective medium supplemented with ampicillin (100 ug/ml), arabinose (0.2%) and sucrose (5%). The agar trays were poured using an agar-autoclave and pump (Integra, Switzerland) to minimise tray-to-tray variation in agar colour and depth. After plating, the colonies were grown by incubating the trays at 37°C for 18 to 24 hours. The *E.coli* cells expressed fusion proteins under the control of the arabinose promoter, and those cells expressing single fusion proteins able to auto-activate the sacB reporter gene were unable to grow, since expression of the sacB gene confers sensitivity to sucrose supplemented in the growth media at high concentrations.

Automated picking of *E.coli* clones for DNA analysis using vision-controlled robotic systems such as described in Lehrach et al. (1997) is well known in the art. Such systems should also be appropriate for the analysis of *E.coli* cells that express interacting or potentially interacting fusion proteins. Therefore, a laboratory picking robot was used to automatically pick individual *E.coli* colonies from the selective agar-trays into individual wells of a sterile 384-well microtiter plate (Genetix, UK) containing sterile liquid medium. The cells expressing the pBAD18- $\alpha$ RNAP fusion library

were inoculated into liquid LB selective medium supplemented with kanamycin (50 ug/ml) and 10% (v/v) glycerol (LB+Kan/10%Gly), while the cells expressing the pBAD30-cI fusion library were inoculated into LB selective medium supplemented with ampicillin (100 ug/ml) and 10% (v/v) glycerol (LB+Amp/10%Gly). The resulting microtiter plates were incubated at 37°C for 18 to 24 hours, and after growth of *E.coli* strains within the microtiter plates, each plate was labelled with a unique number and barcode. The plates were also replicated to create two additional copies using a sterile 384-pin plastic replicator (Genetix, UK) to transfer a small amount of cell material from each well into pre-labelled 384-well microtiter plates and pre-filled with the liquid selective medium containing 10% glycerol as was appropriate for the *E.coli* strain. The replicated plates were incubated at 37 °C for 18 to 24 hours, subsequently labelled, frozen and stored at -70°C together with the original picked microtiter plates of the libraries of *E.coli* cells expressing fusion proteins.

In this manner, we generated a regular grid patterns of *E.coli* cells expressing fusion proteins using a robotic and automated picking system. 384-well microtiter plates have a well every 4.5 mm in a 16 by 24 well arrangement. Therefore, for each 384-well microtiter plate we automatically created a regular grid pattern at a density greater than 4 clones per square centimetre. It will be clear that higher density regular grid-patterns of such an interaction library can be easily generated by a person skilled in the art from these microtiter plates of *E.coli* cells by following the methods disclosed in sections 3.2, 3.3 and 3.4 of this invention. For example, densities of greater than 19 clones per square centimetre can be obtained by robotic pipetting of clones into wells of a 1536-well microtiter plate.

Visual differentiation against false positive clones and the automated creation of a regular grid-pattern of *E.coli* cells expressing a fusion protein

To demonstrate that visual differentiation against cells that express single fusion proteins that auto-activate the readout system could be applied to a prokaryotic two-hybrid system, the libraries of fusion proteins described in section 10.2.1 were utilised. The two transformation mixes were plated onto large 24 x 24 cm agar trays (Genetix, UK) containing selective media. The  $F^-$  cells containing the pBAD18- $\alpha$ RNAP fusion library were plated onto LB selective medium supplemented with kanamycin (50 ug/ml), arabinose (0.2%) and X-Gal (2 ug/ml). The  $F^+$  cells containing the pBAD30-cI fusion library were plated LB selective medium supplemented with ampicillin (100 ug/ml), arabinose (0.2%) and X-Gal (2 ug/ml). The agar trays were poured using an agar-autoclave and pump (Integra, Switzerland) to minimise tray-to-tray variation in agar colour and depth. After plating, the colonies were grown by incubating the trays at 37°C for 18 to 24 hours and to allow any blue colour of colonies to develop. The *E.coli* cells expressed fusion protein under the control of the arabinose promoter, and those cells expressing fusion proteins able to auto-activate the lacZ reporter gene turned blue by enzymatic reaction of the X-Gal substrate as is well known in the art.

Using an automated picking system, white *E.coli* cells expressing single fusion proteins unable to activate the readout system were automatically visually differentiated from false positive *E.coli* cells that had turned blue and only white *E.coli* cells were arrayed in a regular grid pattern. A standard laboratory picking robot (Lehrach et al., 1997) was used except that the improvements relating to reliable sorting of white from blue yeast colonies as described in section 3.1 was also used to reliably discriminate between white and blue *E.coli* colonies. White *E.coli* colonies from the two sets of agar trays prepared above were automatically picked and

inoculated into the appropriate selective media in 384-well microtiter plates as described in section 10.2. It will be recognised by a person skilled in the art that higher density regular grid patterns of these clones may easily be formed.

Automated interaction conjugation to combine genetic elements in *E.coli* cells

It will be clear to a person skilled in the art that automated interaction mating on a solid support as described for yeast cells in section 9.1 is equally appropriate for *E.coli* cells of different conjugation types that have been selected by the methods of genetic preselection or visual differentiation as disclosed in this invention. In such case, appropriate modifications to the selective media would be required. However, a person skilled in the art would be able to recognise and effect said modifications to the selective media by following the disclosures herein.

To demonstrate an automated approach to interaction conjugation based on liquid culture, two libraries of clones that express fusion proteins were prepared as described in section 10.1. The  $F^-$  cells containing the pBAD18- $\alpha$ RNAP fusion library were plated onto LB selective medium supplemented with kanamycin (50 ug/ml), arabinose (0.2%) and sucrose (5%). The  $F^+$  cells containing the pBAD30-cI fusion library were plated LB selective medium supplemented with ampicillin (100 ug/ml), arabinose (0.2%) and sucrose (5%).

To conduct the liquid interaction conjugation, the resulting  $F^-$  and  $F^+$  colonies were separately collected off the agar-trays by washing with 20 ml of liquid LB medium. These two mixtures of *E.coli* clones were carefully resuspended, pelleted and washed with LB. The two populations of cells were combined in 500 ml of LB liquid media and incubated at 37°C with gentle shaking for 6 hours to allow interaction conjugation to proceed. The resulting mixture of *E.coli* cells was pelleted by

gentle centrifugation at 3000 rpm for 5 min, washed twice with 50 ml of LB liquid media and finally, 10 ml of the resulting cell suspension was plated onto each of five 24 x 24 cm agar-trays containing 300 ml of the solid LB selective medium supplemented with ampicillin (100 ug/ml), kanamycin (50 ug/ml), arabinose (0.2%) and tetracycline (35 ug/ml) (LA+Amp+Kan+Tet+ara). The agar trays were poured using an agar-autoclave and pump (Integra, Switzerland) to minimise tray-to-tray variation in agar colour and depth. After plating, the colonies were grown by incubating the trays at 37°C for 18 to 24 hours.

After incubation, resulting *E.coli* cells that expressed interacting fusion proteins grew on the surface of the selective agar, and were automatically picked using a laboratory picking system as described in section 10.2 except that picked clones were inoculated into microtiter plates containing the liquid LB medium supplemented with ampicillin (100 ug/ml), kanamycin (50 ug/ml) and 10% (v/v) glycerol (LB+Amp+Kan/10%Gly). The interaction library comprising the *E.coli* cells contained in the microtiter plates were grown by incubation at 37°C for 18 to 24 hours. Two further copies of the interaction library were made into new microtiter plates containing LB+Amp+Kan/10%Glyc growth medium, all plates were individually labelled with a unique barcode and stored at -70 °C until required for further analysis as described above. It will be recognised by a person skilled in the art that higher density regular grid patterns of these clones may easily be formed.

Generation of a regular grid pattern of clones from an interaction library on planar carriers using automation

A high-throughput spotting robot such as that described by Lehrach et al. (1997) was used to construct porous planar carriers with a high-density regular grid-pattern of *E.coli* clones from the defined interaction library contained within

384-well microtiter plates that is described above. The robot recorded the position of individual clones in the high-density grid-pattern by the use of a pre-defined duplicate spotting pattern and the barcode of the microtiter plate. Individually numbered membrane sheets sized 222 x 222 mm (Hybond N+, Amersham UK) were pre-soaked in LB medium, laid on a sheet of 3MM filter paper (Whatmann, UK) also pre-soaked in LB medium and placed in the bed of the robot. The interaction library was automatically arrayed as replica copies onto the membranes using a 384-pin spotting tool affixed to the robot. Microtiter plates from the first copy of the interaction library were replica spotted in a '5x5 duplicate' pattern around a central ink guide-spot onto 10 nylon membranes - corresponding to positions for over 27,000 clones spotted at a density of over 100 spots per cm<sup>2</sup>. The robot created a data-file in which the spotting pattern produced and the barcode that had been automatically read from each microtiter plate was recorded.

Each membrane was carefully laid onto approximately 300 ml of solid agar media in 24 x 24 cm agar-trays. Six membranes were transferred to LB+Amp+Kan+Tet agar containing 0.2% arabinose and two each of the remaining membranes were transferred to either LB agar supplemented with kanamycin (50 ug/ml), arabinose (0.2%) and tONPG (1 mM) (LB+kan+ara+tONPG) or LB agar supplemented with ampicillin (100 ug/ml), arabinose (0.2%) and streptomycin (at an appropriate concentration for counterselction) (LB+Amp+ara+Sm). The *E.coli* colonies were allowed to grow on the surface of the membrane by incubation at 37 °C for 18 to 24 hours.

Detection of the readout system in a regular grid pattern

Two membranes from each of the selective media was processed to detect  $\beta$ -galactosidase activity using the method of Breeden & Nasmyth (1985) and a digital image was captured and stored on computer as described in section 4.1. Using the image analysis and computer systems described section 4.1, positive

*E.coli* clones were identified by consideration of the activation state of the  $\beta$ -galactosidase readout system when clones had been grown on the various selective media. Positive clones were identified as those that turned blue after growth on the selective media LB+Amp+Kan+Tet+ara but not when grown on either of the counterselective media LB+Kan+ara+tONPG or LB+Amp+ara+Sm.

#### Identification of individual members of the interaction

A positive *E.coli* clone (identified as 15F09) that expressed interacting fusion proteins as determined by the computer systems as described above, was recovered from a stored frozen copy of the interaction library. Both members comprising the interaction were recovered by specific PCR amplification of the insets carried by the pBAD18- $\alpha$ RNAP and pBAD30-cI plasmids directly from the *E.coli* culture using plasmid-specific primers. Both members of the interaction were sequenced by standard procedures, and the information entered into a database as described in Example 7.

As described in section 4.1, high-density arrays of DNA representing interaction libraries or members comprising interaction libraries can be made by transfer to solid supports by a variety of means. To demonstrate the applicability of DNA hybridisation to identify *E.coli* clones carrying plasmids that encode for interacting fusion proteins, one membrane that had been taken from the LB+Amp+Kan+Tet+ara growth medium was processed to affix the DNA carried by the *E.coli* cells comprising the interaction library according to the method of Hoheisel et al (1991). The insert carried by the pBAD30-cI plasmid of clone 15F09 was radioactively labelled by the method of Feinberg & Vogelstein, (1983) and used as a hybridisation probe to the DNA array, and positive signals identified as described in section 4.1. A clone (22C11) was identified as hybridising to the probe and was shown to be a positive clone by query of the data based described in section



4.1. In this manner, further steps in a protein-protein interaction pathway can be identified by hybridisation, consideration of reporter gene activation of hybridisation-positive clones and recovery of plasmids encoding members comprising these interactions. Recovery of the plasmids allows further investigation such as DNA sequencing to identify the members or repeated hybridisation to identify further steps in the protein-protein interaction pathway and hence develop protein-protein interaction networks as described in section 6.6.

**Example 11:** Application of the improved two-hybrid system to a mammalian two-hybrid system

#### 11.1 Strains, readout systems and vectors

The human embryonic kidney fibroblast-derived cell line HEK 293 (or simply 293 cells) is especially suitable for mammalian 2H studies due to its high susceptibility for DNA during transfection (Graham, F.L. and Van der Eb, A.J. (1973), *Virology* 54: 536-539; Graham, F.L., Smiley, J., Russell, W.C. and Nairn, R. (1977), *J. Gen. Virol.* 36: 59-72). The cell line is available from ATCC.

Plasmids carrying the mammalian readout systems named pG5E1bEGFPneo, pG5E1bEGFP<sub>hyg</sub> or pG5E1bEGFP<sub>pur</sub> are used. These plasmids contain the TATA element of the adenoviral E1b gene and five tandem copies of the GAL4 responsive element UAS<sub>G</sub> (5'-CGGAGTACTGTCC TGCG 3') (Sadowski, I., Ma, J., Treizenberg, S. and Ptashne, M. (1988), *Nature* 335: 559-560) positioned immediately upstream of the coding sequence for the enhanced green fluorescent protein (EGFP; Yang, T.T., Cheng, L. and Kain, S.R. (1996), *Nucl. Acids Res.* 24 (22): 4592-4593). These reporter plasmids are generated by replacing the coding sequence for CAT in G5E1bCAT (Dang, C.V., Barrett, J., Villagarcia, M., Resar, L.M.S., Kato, G.J. and Fearon, E.R. (1991), *Mol. Cell. Biol.* 11: 954-962) by the EGFP coding sequence and

introducing either a neomycin, hygromycin or puromycin resistance marker gene ( $\text{neo}^r$ ,  $\text{hyg}^r$  or  $\text{pur}^r$ ) using standard subcloning procedures.

The plasmids pMneo1,2,3 or pMhyg1,2,3, which are derived from pM1,2,3 (Sadowski, I., Bell, B., Broad, P. and Hollis, M. (1992), Gene 118: 137-141) by insertion of either  $\text{neo}^r$  or  $\text{hyg}^r$  marker gene using standard subcloning procedures, are series (1,2,3 correspond to three possible reading frames) of improved Gal4p-fusion vectors derived from the pSG424 plasmid, which was designed for mammalian expression of fusion proteins that contain the DNA-binding domain of the yeast Gal4 protein (Sadowski, I. and Ptashne, M. (1989), Nucl. Acids Res. 17: 7539). This vector contains a polylinker preceded by coding sequences for Gal4p amino acids 1-147. Thus, a hybrid reading frame that encodes a Gal4p-fusion protein can be generated by inserting cDNA sequences into the polylinker region of pSG424/pM's. Transcripts of the hybrid reading frame are initiated from the SV40 early promoter and their processing is facilitated by the SV40 polyadenylation signal. Alternatively, the hybrid reading frames are subcloned into pLXSN or any other similar retroviral vector to allow packaging cell line-aided infection of target cells.

The plasmids pVP-Nconeo and pVP-Ncohyg are derived from pVP-Nco vector (Tsan, J., Wang, Z., Jin, Y., Hwang, L., Bash, R.O., Baer, R. The Yeast Two-Hybrid System, edn 1. Edited by Bartel, P.L., Fields, S. New York: Oxford University Press (1997): 217-232) by insertion of either a  $\text{neo}^r$  or  $\text{hyg}^r$  marker gene using standard subcloning procedures. pVP-Nco in turn is an improved version of the pNLVP16 plasmid, which was constructed for the expression of herpes simplex virus protein VP16-fusion proteins in mammalian cells (Dang, C.V., Barrett, J., Villa-Garcia, M., Resar, L.M.S., Kato, G.J. and Fearon, E.R. (1991), Mol. Cell. Biol. 11: 954-962). A polylinker sequence is preceded by an artificial reading frame including the eleven amino-terminal residues of Gal4p (MKLLSSIEQAC), a

nuclear localization signal from the SV40 large T antigen (PKKKRKVD) and the acidic transactivation domain (amino acids 411-456) of the VP16 protein. Alternatively, the hybrid reading frames comprising Gal4 (1-147) and individual sequences of a cDNA library are subcloned into pLXSN or any other similar retroviral vector to allow packaging cell line-aided infection of target cells.

### 11.2 Detection and Identification of Interacting Proteins

A number of monoclonal 293 cell lines stably containing the pG5E1bEGFPneo-, pG5E1bEGFP<sub>hyg</sub> or pG5E1bEGFP<sub>pur</sub> readout system are generated by the method of calcium phosphate transfection (Chen, C. and Okayama, H. (1987), Mol. Cell. Biol. 7:2745-2752), lipofectamine transfection or any other common transfection method, followed by selection in G418, hygromycinB (HygB) or puromycin containing medium, respectively. It is tested subsequently which particular clone is most appropriate (number of readout system copies and site(s) of integration into the host chromosomes may influence expression levels and inducibility of the reporter gene) for the method of invention.

The selected 293-G5E1bEGFPneo, 293-G5E1bEGFP<sub>hyg</sub> or 293-G5E1bEGFP<sub>pur</sub> reporter cell line is used as a "modified host cell strain" to perform the method of invention (detection and identification of interacting proteins).

Two pools representing all three reading frames of the two vector series pMneo or M<sub>hyg</sub> and pVP-Ncone or pVP-Nco<sub>hyg</sub> were prepared by Not 1/Sal 1 digestion and pooling of 1 µg each of vectors pMneo / pM<sub>hyg</sub> 1,2,3 and pVP-Ncone / pVP-Nco<sub>hyg</sub> 1,2,3 respectively. 300 ng of a cDNA insert mixture that was isolated as described in section 6.1 was split into two equal fractions and was ligated with 50 ng of each prepared vector-series pool. Following ligation, each reaction was then separately transformed into electro-competent *E.coli* cells,

and recombinant clones for each library were selected on five 24 x 24 cm plates ampicillin. Approximately 500  $\mu$ g of the pVP-Nconeo / pVP-Ncohyg and 500  $\mu$ g of the pMneo / pMhyg libraries were extracted from *E.coli* transformants by washing off the plated cells and a subsequent QiaPrep plasmid extraction of the wash mixture as described above. 16  $\mu$ g of each vector was used to transfect a 10cm plate of 293 cells.

### 11.3 Pre-selection against False Positives by visual differentiation

The pMneo1,2,3 or pMhyg1,2,3 plasmids containing the cDNA library fused to the Gal4-DNA binding domain were transfected into the selected 293 reporter cell line. For infection with retroviruses, designated packaging cell lines are transfected with the respective retroviral vectors and virus-containing supernatant from such cultures is then used to infect the reporter cell line (according to standard protocols; e.g. Redemann, N., Holzmann, v.Rüden, T., Wagner, E.F., Schlessinger, J. and Ullrich, A. (1992), Mol. Cell. Biol. 12: 491-498). Transfection and infection protocols can be optimized in a way to introduce on average only one plasmid per cell by adjusting the plasmid concentration for transfection or the virus titer during infection. Antibiotics G418 or HygB are employed to select for successfully transfected/infected reporter cells.

At this stage it is necessary to eliminate those cells that display a readout system activation as a consequence of only expressing a DNA-binding domain-fusion protein (in which case the fusion protein would be referred to as an "auto-activator"), instead of requiring an appropriate (interacting) transactivation domain-fusion protein to be coexpressed. Thus, the resultant polyclonal pool of stably transfected/infected reporter cells is then subjected to a preselection screen using the readout system to visually differentiate cells that express auto-activating fusion proteins. In the EGFP-based

readout system cells expressing auto-activators can be identified by screening for expression of EGFP and consequently for the ability of the respective cells to emit a green fluorescent light (507 nm) upon stimulation with the appropriate excitatory wavelength (488 nm) (Yang, T.T., Cheng, L. and Kain, S.R. (1996), Nucl. Acids Res. 24 (22): 4592-4593). Monitoring readout system activation is either done by eye using a fluorescence microscope or by an automated detection device. The cells that activated the GRP reporter system were visually differentiated and sorted from other cells not activating the reporter system using a fluorescent assisted cell sorting device (FACS). Alternatively, elimination of false positive cells expressing auto-activators is either done manually or by removal/killing of cells by means of a suction pump or a micromanipulator or by a detector-linked automated system employing micromanipulators or a laser ablation device.

After elimination of cells that express autoactivating fusion proteins, the remaining polyclonal pool of 293 reporter cells expressing DNA-binding fusion proteins are then subjected to a second transfection /infection step as described above using pVP-Nconeo or pVP-Ncohyg plasmids or respective retroviral derivatives containing the cDNA library fused to the VP16 transactivator sequence. Selection for successfully transfected/infected cells employing antibiotics G418 or HygB is optional here. If selection is desired it has to be made sure that the resistance marker that forms part of the readout system is different from the marker genes on previously transfected/infected vectors. Addition of the antibiotics selecting for the second transfection/infection-vector may be necessary, if the subsequent screening/final selection procedures take several days to complete, in order to prevent loss/diluting out of the plasmids in the absence of selective pressure. A complete selection also eliminates cells that have not been successfully cotransfected (i.e. have not received a pVP-Nco-plasmid), although such cells would not be a major

problem (as long as transfection/infection efficiency is high) because they would not be identified by the interaction screening anyway. It is also noteworthy that the longer the cells are kept in culture until cell lysis (and molecular analyses of the interacting proteins and their corresponding cDNA sequences) the more likely it is to lose cDNAs that encode for more or less toxic fusion proteins.

#### 11.4 Automated Identification of Cells Expressing Interacting Proteins

The resulting polyclonal pool of doubly transfected reporter cells is then subjected to visual screening for interacting proteins as described for the visual preselection. Green fluorescent ("positive") cells, indicative of the expression of two interacting proteins were automatically sorted using a FACS system to arrange cells in a regular grid pattern in wells of a microtitre plate. Subsequently, single cell PCR and DNA sequencing was conducted to identify members comprising the interactions. Alternatively, the positive cells can be seeded onto a culture dish in a regular array/grid pattern. Cells might also be placed one by one into small wells of a multiwell dish and provided with an appropriate growth factor-supplemented medium or conditioned medium to allow the cells to survive and grow in isolation from other cells.

#### 11.5 Double Preselection and Cell Fusion

The cotransfection protocol described above only includes a single preselection (instead of a double preselection). It does not include the possibility of a preselection against false positive clones arising from pVP-Nco (transactivation domain-cDNA fusion library) plasmids. Although the number of false positives from pVP-Nco plasmids is usually much lower than from pM1,2,3 (DNA binding domain-cDNA fusion library) plasmids, it may under some circumstances be necessary to apply a double preselection strategy.

To that end two different polyclonal pools of stable cell lines expressing either members of the pM- or pVP-Nco-cDNA fusion library are generated by transfection/infection of the 293 reporter cell line and selected by means of the respective (different) antibiotics (G418 and HygB) as described above. Both pools of cell lines are then subjected separately to preselection and elimination of false positive clones as detailed above.

In order to combine both fusion vectors and their corresponding expressed fusion proteins in one cell, individual cells of both pools of cell lines are fused together using state-of-the-art cell fusion-protocols involving PEG-facilitated electrofusion as described in Li, L.-H. and Hui, S.W. (1994), *Biophys. J.* 67: 2361-2366; Hui, S.W., Stoicheva, N. and Zhao, Y.-L. (1996), *Biophys. J.* 71: 1123-1130, and Stoicheva, N. and Hui, S.W. (1994), *Membrane Biol.* 140: 177-182. Fusions between one cell of both pools is desired. For that purpose one cell of each pool is placed into each well of a multiwell dish as detailed above. After cell fusion, the combined cells are then subjected to visual selection. Cells are left on the same dish for visual or automated screening or collected and sorted by FACS.

#### 11.6 Double Preselection and Cell Fusion Using an Inducible Expression System

A disadvantage of the above described double preselection method is that proteins with toxic or anti-proliferative effects and their corresponding cDNAs will be lost during the lengthy selection process necessary to establish polyclonal pools of stable cell lines for both cDNA-fusion library-sequences. In order to prevent elimination of cDNA sequences encoding for toxic/ anti-proliferative proteins one can combine the double preselection strategy with the following inducible system.

The host cell strain is a 293 cell line which expresses a tetracycline (Tet)-controlled transactivator (tTA), which is a fusion of amino acids 1-207 of the tetracycline repressor (TetR) and the C-terminal activation domain (130 amino acids) of herpes simplex virus protein VP16. The cell line is called 293 Tet-Off as tTA is able to activate transcription from a Tet operator sequence (tetO)-controlled gene only in the absence of Tet. The reverse situation exists in the 293 Tet-On cell line, which stably expresses a reverse tTA ((r)tTA) that requires the presence of Tet to induce transcription from tetO-regulated genes. Both, 293 Tet-Off and 293 Tet-On cell lines are G418-resistant (neo<sup>r</sup>). These cell lines are available through Clontech Inc.. tTA plasmids used to generate 293 Tet-Off and 293 Tet-On cell lines are described in Gossen, M. and Bujard, H. (1992), Proc. Natl. Acad. Sci. USA 89: 5547-5551 and in Gossen M., Freundlieb, S., Bender, G., Müller, G., Hillen, W. and Bujard, H. (1995), Science 268: 1766-1769.

293 Tet-On or -Off cell lines are then transfected with a readout system (described in 11.1.) and the reporter cell lines 293 Tet-On- or -Off-pG5E1bEGFP<sub>hyg</sub>/pur are generated through selection in G418 or HygB.

The sequences for the Gal4-DNA binding domain and for the SV40 nuclear localisation signal/VP16 transactivation domain (details and references as given in 11.1) are retrieved from pM and pVP-Nco plasmids and separately subcloned into the polylinker of pREV-TRE, a retroviral vector (Clontech Inc.) to generate pREV-TRE-Gal4 and pREV-TRE-VP16. pREV-TRE contains the retroviral extended packaging signal,  $\Psi$ <sup>+</sup>, which allows for production of infectious but replication-incompetent virus in conjunction with a packaging cell line such as PT67, followed by a hyg<sup>r</sup> gene (selectable marker) and seven copies of tetO fused to the cytomegalovirus (CMV) minimal promoter immediately 5' of the polylinker.  $\Psi$ <sup>+</sup> and polylinker sequences



are flanked by 5' and 3' LTRs, respectively. pREV-TRE is available from Clontech Inc.. cDNA libraries are subcloned into the polylinker of pREV-TRE.

The above described reporter cell lines are separately infected with either pREV-TRE-Gal4- or pREV-TRE-VP16-derived retroviral particles. A polyclonal pool of new stable cell lines is selected in both cases using the resistance selection marker gene *hyg<sup>r</sup>*. Transient expression of fusion proteins from pREV-TRE plasmids has to be induced by withdrawal (Tet-Off) or addition (Tet-On) of Tet in order to allow for double preselection and elimination of false positives as described above.

#### 11.7 Cell Fusion and Selection for Cells Expressing Interacting Proteins

The remaining polyclonal pools of cell lines are then subjected to cell fusion as described above. The HygB concentration in the culture medium is increased to minimize a possible loss of either one component of the pairs of fusion protein cDNA sequences present in all fused cells. For the detection of positive clones, i.e. cells expressing a pair of interacting proteins (as detailed above), expression of fusion proteins has to be induced by addition or withdrawal of Tet.

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**Table 1**

Oligonucleotide adapters for the construction of the novel yeast two-hybrid vectors pBTM118 a, b and c and pGAD428 a, b and c.

Oligonucleotide	Sequence (5'-3')
a sense	TCGAGTCGACGCGGCCGCTAA
A antisense	GGCCTTAGCGGCCGCGTCGAC
b sense	TCGAGGTCGACGCGGCCGAGTAA
B antisense	GGCCTTACTGCGGCCGCGTCGACC
c sense	TCGAGAGTCGACGCGGCCGCTTAA
c antisense	GGCCTTAAGCGGCCGCGTCGACTC

Table 2

Two-hybrid vectors used for the expression of fusion proteins.

Plasmid	Fusion-protein	Insert (kb)	Counter-selection	Selection in yeast	Fusion protein Reference
PBTM117c	LexA	-	CAN1	TRP1	N/A
pBTM117c-HD1.6	LexA-HD1.6	1.6	CAN1	TRP1	Wanker et al., 1997
pBTM117c-HD3.6	LexA-HD3.6	3.6	CAN1	TRP1	Wanker et al., 1997
pBTM117c-SIM1	LexA-SIM1	1.1	CAN1	TRP1	Probst et al., 1997
pBTM117c-MJD	LexA-MJD	1.1	CAN1	TRP1	this work
pBTM117c-HIP1	LexA-HIP1	1.2	CAN1	TRP1	this work
PGAD427	GAL4ad	-	CYH2	LEU2	N/A
pGAD427-ARNT	GAL4ad-ARNT	1.4	CYH2	LEU2	Probst et al., 1997
pGAD427-HIP1	GAL4ad-HIP1	1.2	CYH2	LEU2	Wanker et al., 1997
pGAD427-HIPCT	GAL4ad-HIPCT	0.5	CYH2	LEU2	Wanker et al., 1997
pGAD427-14-3-3	GAL4ad-14-3-3	1.0	CYH2	LEU2	this work
pGAD427-LexA	Gal4ad-LexA	1.2	CYH2	LEU2	this work



Table 3

Yeast strains used for the 5-FOA counterselection and the automated interaction mating

Strain	Plasmids	Selected on
x1a	pBTM117c / pLUA	SD-trp-ade
x2a	pBTM117c-SIM1 / pLUA	SD-trp-ade
x3a	pBTM117c-HIP1 / pLUA	SD-trp-ade
y1 $\alpha$	pGAD427 / pLUA	SD-leu-ade
y2 $\alpha$	pGAD427-ARNT / pLUA	SD-leu-ade
y3 $\alpha$	pGAD427-LexA / pLUA	SD-leu-ade

Table 4

Identification of fusion proteins that activate the URA3 readout system.

a.

Strain	Plasmids	SD-trp -ade	SD-trp -ade+5- FOA	SD-trp -ade- ura
x1a	pBTM117c / pLUA	+	+	-
x2a	pBTM117c-SIM1 / pLUA	+	+	-
x3a	pBTM117c-HIP1 / pLUA	+	-	+

SD-trp-ade: Selective medium lacking tryptophan and adenine.

SD-trp-ade+5-FOA: Selective medium containing 0.2 % 5-FOA.

SD-trp-ade-ura: Selective medium lacking tryptophan, adenine and uracil.

b.

Strain	Plasmids	SD-leu -ade	SD-leu -ade+5-FOA	SD-leu -ade-ura
y1α	pGAD427 / pLUA	+	+	-
y2α	pGAD427 -ARNT/pLUA	+	+	-
y3α	pGAD427 -LexA/pLUA	+	-	+

SD-leu-ade: Selective medium lacking leucin and adenine.

SD-leu-ade+5-FOA: Selective medium containing 0.2 % 5-FOA.

SD-leu-ade-ura: Selective medium lacking leucin, adenine and uracil.

**Table 5**

Identification of fusion proteins that activate the LacZ readout system.

A. L40ccu yeast cells transformed with pBTM117c plasmid constructs expressing a fusion protein comprising the LexA DNA binding domain are plated on minimal medium lacking trptophan, buffered to pH 7.0 with potassium phosphate and containing 2 ug/ml of X-Gal (SD-trp/XGAL): Results for the state of the readout system for various auto-activating and non-auto-activating fusion proteins

Plasmid Construct	Fusion protein	Growth on SD-trp/XGAL	Blue colouration
pBTM117c	LexA	+	-
pBTM117c-HD1.6	LexA-HD1.6	+	-
pBTM117c-HD3.6	LexA-HD3.6	+	-
pBTM117c-SIM1	LexA-SIM1	+	-
pBTM117c-MJD	LexA-MJD	+	-
pBTM117c-HIP1	LexA-HIP1	+	+

B. L40ccu $\alpha$  yeast cells transformed with pGAD427 plasmid constructs expressing a fusion protein comprising the GAL4ad activation domain are plated on minimal medium lacking leucine, buffered to pH 7.0 with potassium phosphate and containing 2 ug/ml of X-Gal (SD-leu/XGAL): Results for the state of the readout system for various auto-activating and non-auto-activating fusion proteins.

Plasmid Construct	Fusion protein	Growth on SD-leu/XGAL	Blue colouration
pGAD427	GAL4ad	+	-
PGAD427-ARNT	GAL4ad-ARNT	+	-
PGAD427-HIP1	GAL4ad-HIP1	+	-
PGAD427-HIPCT	GAL4ad-HIPCT	+	-
PGAD427-14-3-3	GAL4ad-14-3-3	+	-
PGAD427-LexA	Gal4ad-LexA	+	+

## CLAIMS

1. A method for the identification of at least one member of a pair or complex of interacting molecules, comprising:
  - (a) providing host cells containing at least two genetic elements with different selectable and counter-selectable markers, said genetic elements each comprising genetic information specifying one of said members, said host cells further carrying a readout system that is activated upon the interaction of said molecules;
  - (b) allowing at least one interaction, if any, to occur;
  - (c) selecting for said interaction by transferring progeny of said host cells to:
    - (ca) at least two different selective media, wherein each of said selective media allows growth of said host cells only in the absence of at least one of said counter-selectable markers and in the presence of a selectable marker; and
    - (cb) a further selective medium that allows identification of said host cells only on the activation of said readout system;
  - (d) identifying host cells containing interacting molecules that:
    - (da) do not activate said readout system on any of said selective media specified in (ca); and
    - (db) activate the readout system on said selective medium specified in (cb); and
  - (e) identifying at least one member of said pair or complex of interacting molecules

2. The method of claim 1 wherein said pair or complex of interacting molecules is selected from the group consisting of RNA-RNA, RNA-DNA, RNA-protein, DNA-DNA, DNA-protein, protein-protein, protein-peptide, or peptide-peptide interactions.
3. The method of claim 1 or 2 wherein said genetic elements are plasmids artificial chromosomes, viruses or other extra chromosomal elements.
4. The method of any one of claims 1 to 3 wherein said interactions lead to the formation of a functional transcriptional activator comprising a DNA-binding and a transactivating protein domain and which is capable of activating a responsive moiety driving the activation of said readout system.
5. The method of claim 4 wherein said readout system is a detectable protein.
6. The method of claim 5 wherein said detectable protein is encoded from at least one of the genes *lacZ*, *HIS3*, *URA3*, *LYS2*, *sacB*, *tetA*, *gfp* or *HRPT*.
7. The method of any one of claims 1 to 6 wherein said host cells are yeast cells, bacterial cells, mammalian cells, insect cells or plant cells.
8. The method of any one of claims 1 to 7 further comprising transforming or transfecting said host cells with said genetic elements prior to step (a).
9. The method of any one of claims 1 to 8 wherein cell fusion, conjugation or interaction mating is used for the generation of said host cells with said genetic elements prior to step (a).

10. The method of any one of claims 1 to 9 wherein said counter-selectable markers selected against in step (ca) are selected from the group of CAN1, CYH2, LYS2, URA3, *lacY*, *rpsL* HPRT and *sacB*.
11. The method of any one of claims 1 to 10 wherein said selectable marker is an auxotrophic or antibiotic marker.
12. The method of claim 11 wherein said auxotrophic or antibiotic marker is LEU2, TRP1, URA3, ADE2, HIS3, LYS2 or Zeocin.
13. The method of any one of claims 1 to 12 wherein progeny of host cells of step (b) are transferred to storage compartment.
14. The method of claim 13 wherein said transfer is effected or assisted by automation or a picking robot.
15. The method of claim 13 or 14 wherein said storage compartment comprises an anti-freeze agent.
16. The method of any one of claims 3 to 15 wherein said storage compartment is a microtiter plate.
17. The method of claim 16 wherein said microtiter plate comprises 384 wells.
18. The method of any one of claims 1 to 17 wherein said transfer in step (c) is made or assisted by automation, a spotting robot, pipetting or micropipetting device.
19. The method of claim 18 wherein said transfer is made to a planar carrier.

20. The method of claim 18 or 19 wherein said transfer is in a regular grid pattern of densities of 1 to 1000 clones per  $\text{cm}^2$ .
21. The method of any one of claims 18 to 20 wherein said planar carrier is a membrane.
22. The method of any one of claims 1 to 21 wherein said identification of said host cells in step (d) is effected by visual means from consideration of the activation state of said readout system.
23. The method of any one of claims 1 to 22 wherein said identification of said host cells in step (d) is effected by digital image storage, analysis or processing.
24. The method of any one of claims 1 to 23 wherein said identification of said at least one member of said pair of interacting molecules is effected by nucleic acid hybridization, antibody binding or nucleic acid sequencing.
25. The method of claim 24 wherein said identification made by said antibody reaction or said hybridization is effected using regular grids of said at least one member or of said genetic information encoding said at least one member.
26. The method of claim 25 wherein construction of said regular grids is effected by automation or a spotting robot.
27. The method of any one of claims 24 to 26 wherein said identification is effected by digital image storage, processing or analysis.

28. The method of any one of claims 24 to 27 wherein nucleic acid molecules, prior to said identification, are amplified by PCR or are amplified in as a part of said genetic elements, preferably in bacteria and most preferably in E.coli.
29. The method of any one of claims 1 to 28 wherein, prior to step (a) a preselection against clones that express a single molecule able to activate the readout system is carried out on culture media comprising a counterselective compound.
30. The method of claim 29 wherein said counterselective compound is 5-fluoro orotic acid, canavanine, cycloheximide, sucrose, tONPG, streptomycin or  $\alpha$ -amino-adipate.
31. A method for the production of a pharmaceutical composition comprising formulating said at least one member of the interacting molecules identified by the method of any one of claims 1 to 30 in a pharmaceutically acceptable form.
32. A method for the production of a pharmaceutical composition comprising formulating an inhibitor of the interaction of the interacting molecules identified by the method of any one of claims 1 to 30 in a pharmaceutically acceptable form.
33. A method for the production of a pharmaceutical composition comprising identifying a further molecule of a cascade of interacting molecules, of which the at least one member of said interacting molecules identified by the method of any one of claims 1 to 30 is a part of or identifying an inhibitor of said further molecule.



34. Kit comprising at least one of the following:

- (f) host cells as identified in any of the preceding claims and at least one genetic element comprising said genetic information specifying at least one of said possibly interacting molecules containing a counterselectable marker and specified in any of the preceding claims;
- (g) host cells as identified in any of the preceding claims and at least one genetic element not comprising genetic information specifying at least one of said potentially interacting molecules containing a counterselectable marker and specified in any of the preceding claims;
- (h) at least one genetic element comprising said genetic information specifying at least one of said potentially interacting molecules containing a counterselectable marker and specified in any of the preceding claims;
- (i) at least one genetic element not comprising genetic information specifying at least one of said potentially interacting molecules containing a counterselectable marker and specified in any of the preceding claims;
- (j) host cells comprising at least one and preferably at least two of said genetic elements specified in (h) or (i);
- (k) at least one planar carrier carrying nucleic acid or protein from said host cells comprising at least one member of said genetic elements specified in any of the preceding claims wherein said nucleic acid or protein is affixed to said carrier in grid form and optionally solutions to effect hybridization or binding of nucleic acid probes or proteins to said molecules affixed to said grid;
- (l) at least one storage compartment, planar carrier or computer disc comprising or/and characterizing

genetic elements, host cells, storage compartments or carriers identified in any of (f) to (k); and/or  
(m) at least one yeast strain comprising a *can1* and a *cyh2* mutation.

35. The kit of claim 34, wherein said host cells of (f), (g) or (j) are contained in at least one storage compartment.

36. The kit of claim 34 or 35, wherein said genetic information or said potentially interacting molecules encoded by said genetic information as specified in (i) or (iii) are contained in at least one storage compartment.

37. A computer implemented method for, storing and analysing data relating to potential members of at least one pair or complex of interacting molecules encoded by nucleic acids originating from biological samples, said methods comprising

- (n) retrieving from a first data-table information for a first nucleic acid, wherein said information comprises:
  - (oa) a first combination of letters and/or numbers uniquely identifying the nucleic acid, and
  - (ob) the type of genetic element comprising said nucleic acid and
  - (oc) a second combination of letters and/or numbers uniquely identifying a clone in which a potential member encoded by said nucleic acid was tested for interaction with at least one other potential member of a pair or complex of interacting molecules
- (p) using said second combination of letters and/or numbers to retrieve from said first data-table or optionally further data-tables, information identifying additional nucleic acids encoding for said at least one other potential member in step a3).

38. The method of claim 37 further comprising, using said second combination of letters and/or numbers in step (oc) to retrieve from a second data-table further information, where said further information at least comprises the interaction class of said clone, and optionally additional information comprising,
- (q) the physical location of the clone,
  - (r) predetermined experimental details pertaining to creation of said clone, including at least one of,
  - (ra) tissue, disease-state or cell source of the nucleic acid,
  - (rb) cloning details, and
  - (rc) membership of a library of other clones,
39. The method of claim 38 further comprising, using said information of step (o) on said first and/or of step (p) on additional nucleic acids to relate to a third data-table further characterising said first and/or additional nucleic acids, where said further characterising comprises at least one of
- (s) hybridization data;
  - (t) oligonucleotide fingerprint data;
  - (u) nucleotide sequence;
  - (v) in-frame translation of the said nucleic acids;
  - (w) tissue, disease-state or cell source gene expression data; and
- optionally identifying the protein domain encoded by said first or additional nucleic acids.
40. The method of claim 39 further comprising, identifying if said potential members encoded by the nucleic acids interact, by considering said interaction class of said clone in which nucleic acids were tested for said interaction in step (oc)

41. The method of one of claims 37 to 40, wherein said data relates to 10 to 100 potential members, preferably 100 to 1000 potential members., more preferably 1000 to 10000 potential members and most preferably more than 10,000 potential members.
42. The method of one of claims 37 to 41, wherein said data was generated by the method of claims 1 to 36.
43. The method of claims 38 to 42, wherein said interaction class comprises one of the following:
  - (x) Positive
  - (y) Negative
  - (z) False Positive
44. The method of one of claims 40 to 43 wherein sticky proteins are identified by consideration of the number of occurrences a given member is identified to interact with many different members in different clones of said positive interaction class.
45. The method of one of claims 37 to 44, wherein said first data-table forms part of a first database, and said second and third data tables form part of at least a second database.
46. The method of claim 45, wherein said second database is held on a computer readable memory separate from the computer readable memory holding said first database, and said database is accessed via a data exchange network.
47. The method of claim 46, wherein said second database comprises nucleic acid or protein sequence, secondary or tertiary structure, biochemical, biographical or gene expression information.

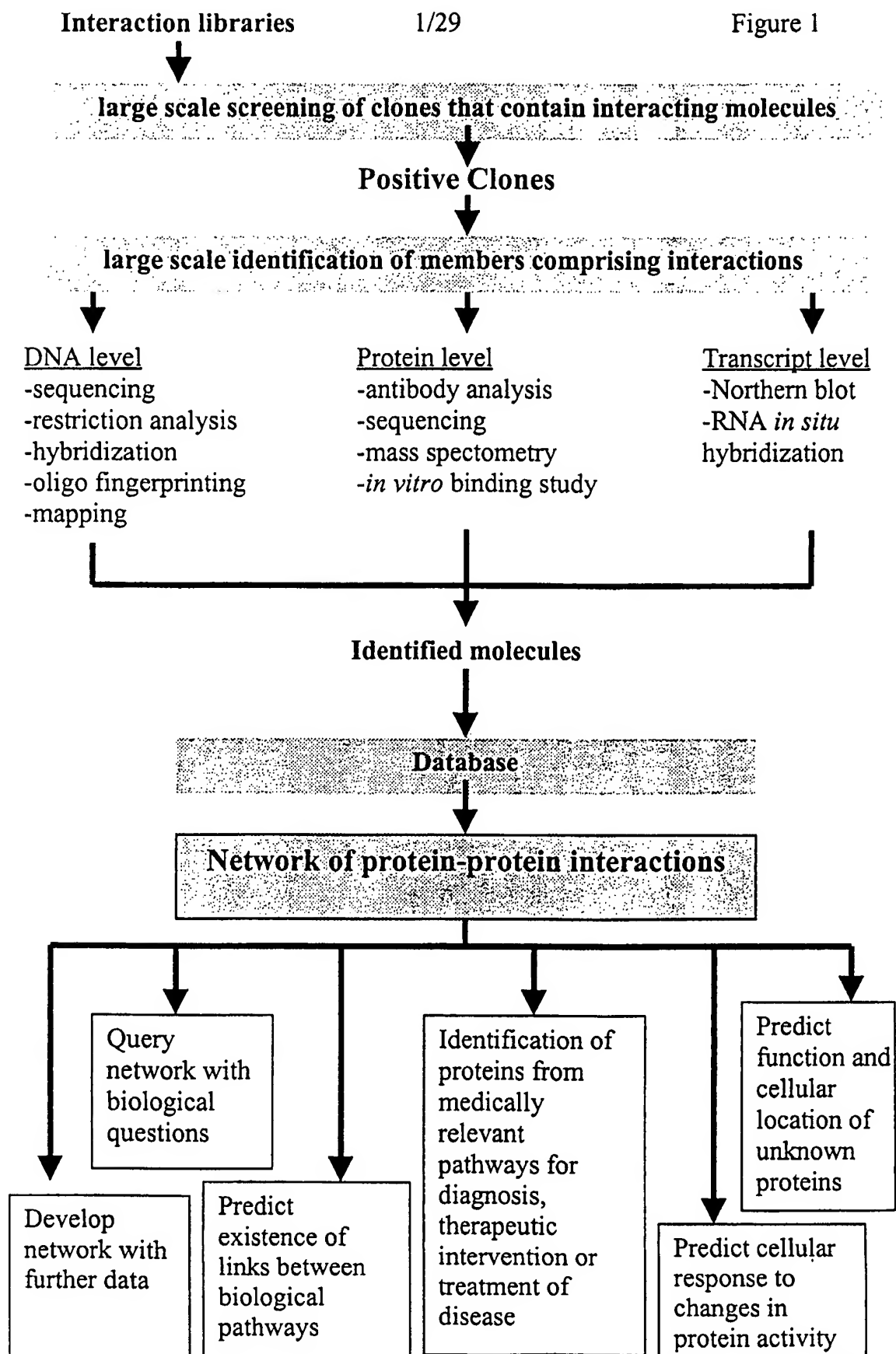
48. The method of claims 37 to 47, wherein data entry to said first, second or further data tables is controlled automatically from said first data base by access to other computer data, programs or computer controlled robots.
49. The method of one of claims 37 to 48, wherein at least one workflow management system is built around particular sets of data to assist in the progress of the method of claims 1 to 36.
50. The method of claim 49, wherein said workflow management system is software to assist in the progress of the identification of members of a pair or complex of interacting molecules using the method of hybridization as specified in claims 24 to 28
51. The method of claims 37 to 50, wherein said data are investigated by queries of interest to an investigator.
52. The method of claim 51, wherein said queries include at least one of,
- (aa) identifying the interaction or interaction pathway between a first and second member of an interaction network
  - (ab) identifying the interaction pathway between a first and second member of an interaction network and through at least one further member,
  - (ac) identifying the interaction or interaction pathway between at least two members characterised by nucleotide acid or protein sequences, secondary or tertiary structures, and
  - (ad) identifying interactions or interaction pathways that are different for said different tissue, disease-state or cell source.
53. The method of claims 51 or 52, wherein parts of said information is stored in a controlled format to assist

data query procedures.

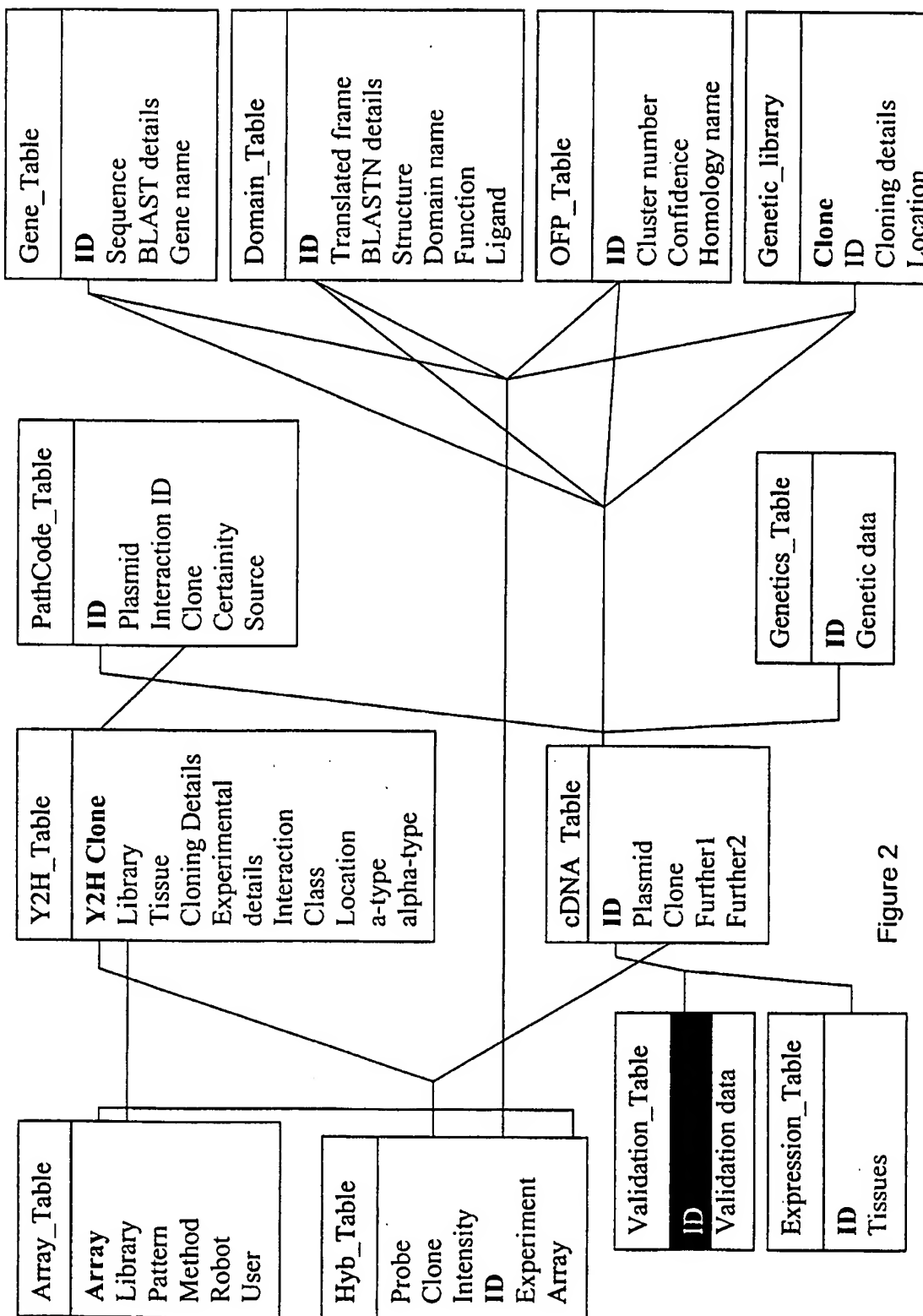
54. The method of claims 51 to 53, wherein the results of said queries are displayed to the investigator in a graphical manner.
55. The method of claims 54, wherein a sub-set of data comprising data characterising nucleic acids identified as encoding members of a pair or complex of interacting molecules of claim 40 is stored in a further data-table or data base.
56. The method of claim 55 wherein consideration of the number of occurrences a given member is identified to interact with a second or further member is used to decide if said data characterising nucleic acids form part of said sub-set of data.
57. The method of claims 55 or 56, wherein additional information or experimental data is used to select those data to form part of said subset.
58. The method of claims 55 to 57, wherein to speed certain data query procedures, the structure in which the data is stored in the computer readable memory is modified.
59. The method of one of claims 37 to 58, wherein the data is held in relational or object oriented data bases.
60. A data storage scheme comprising a data table that holds information on each member of an interaction, where a record in said table represents each member of an interaction, and in which members are indicated to form interactions by sharing a common name.
61. The data storage scheme of claim 60, wherein said common name is a clone name or unique combination of letters and/or numbers comprising said clone name.

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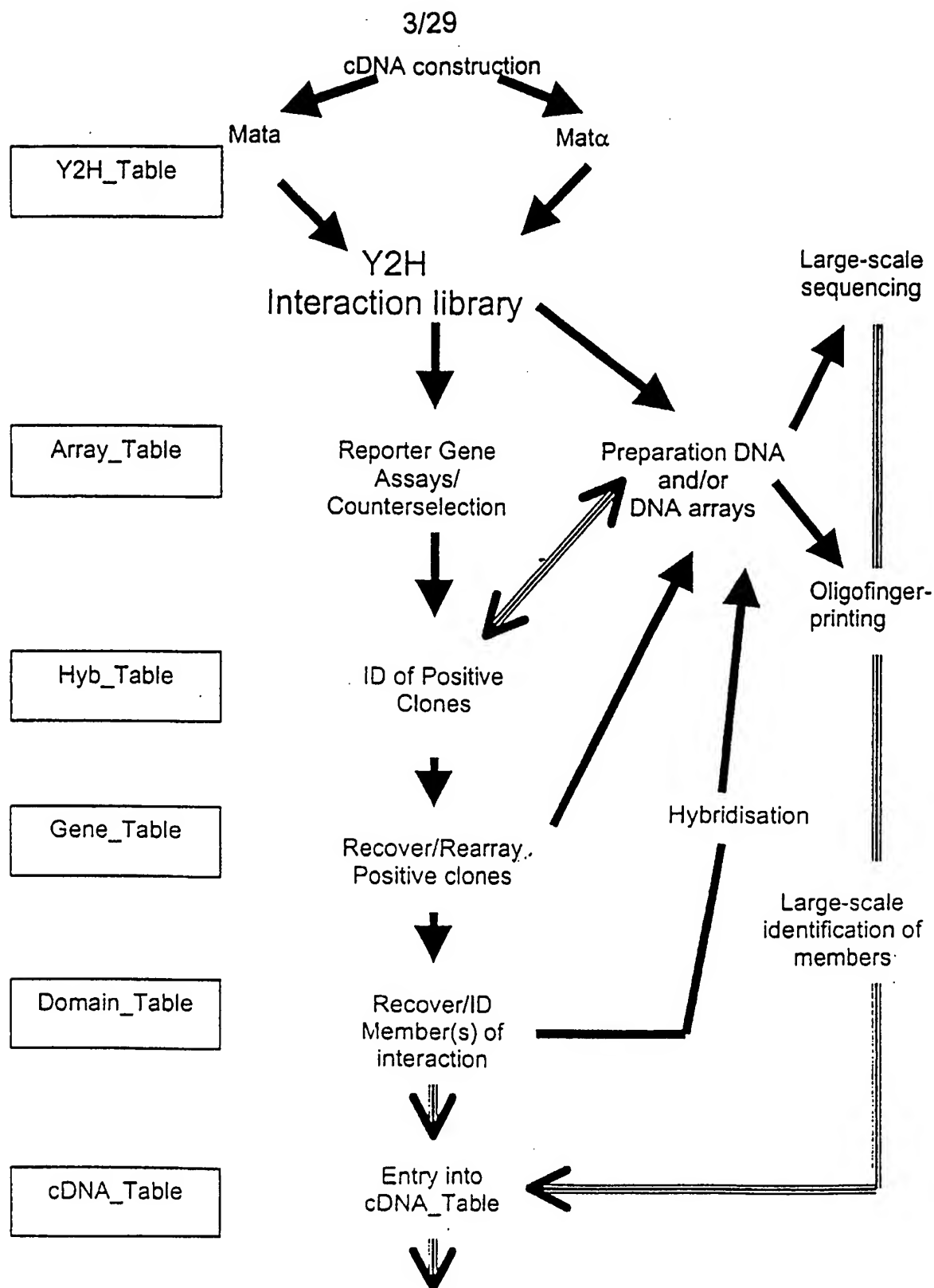
Figure 1



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(continuation from the previous page)

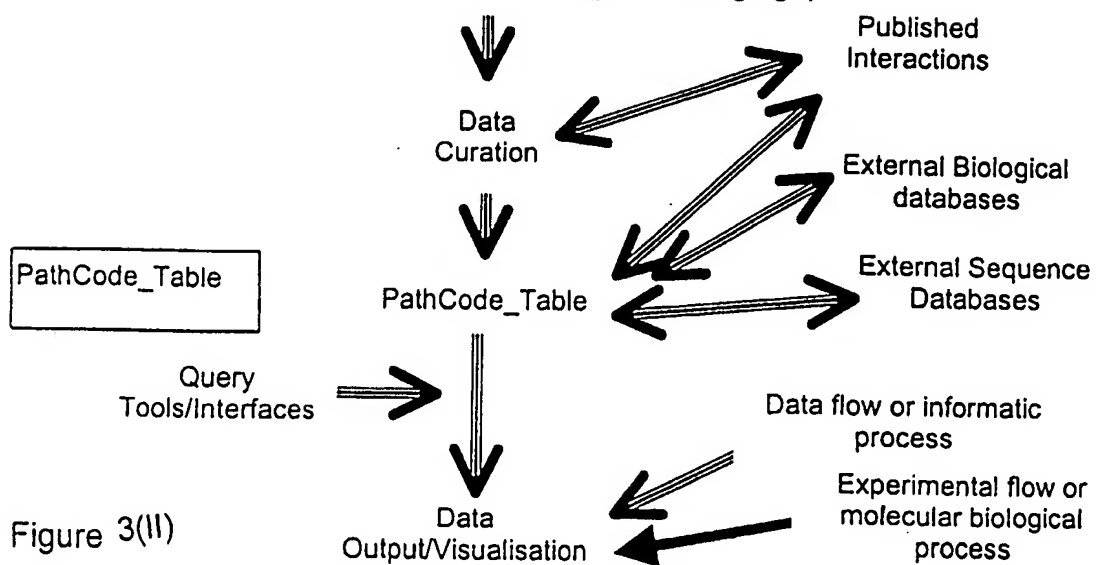
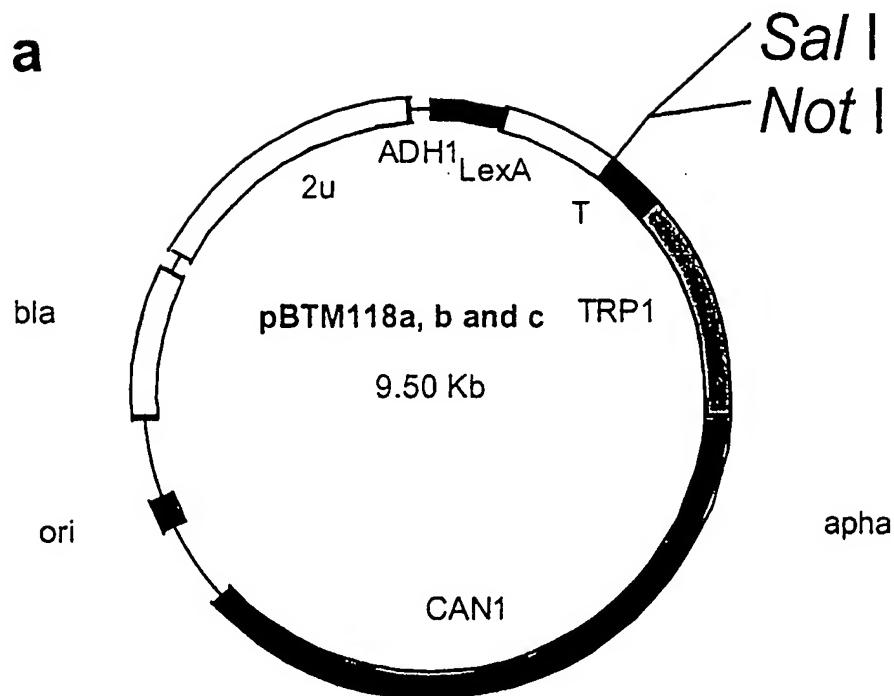


Figure 3(II)

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**a**



**b**

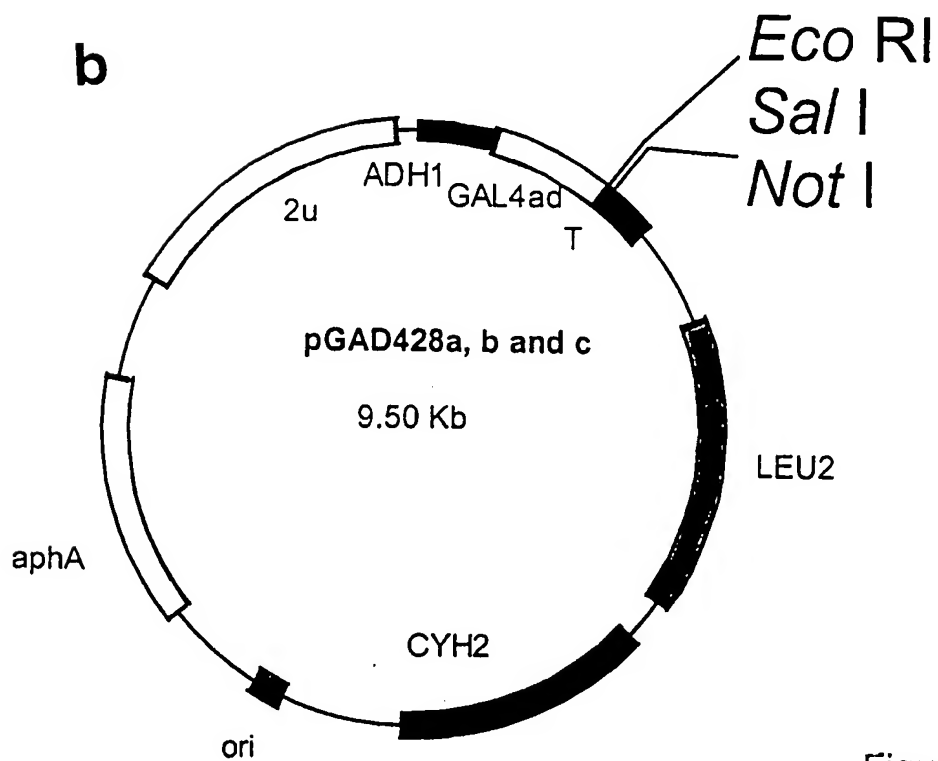


Figure 4 (I)

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b

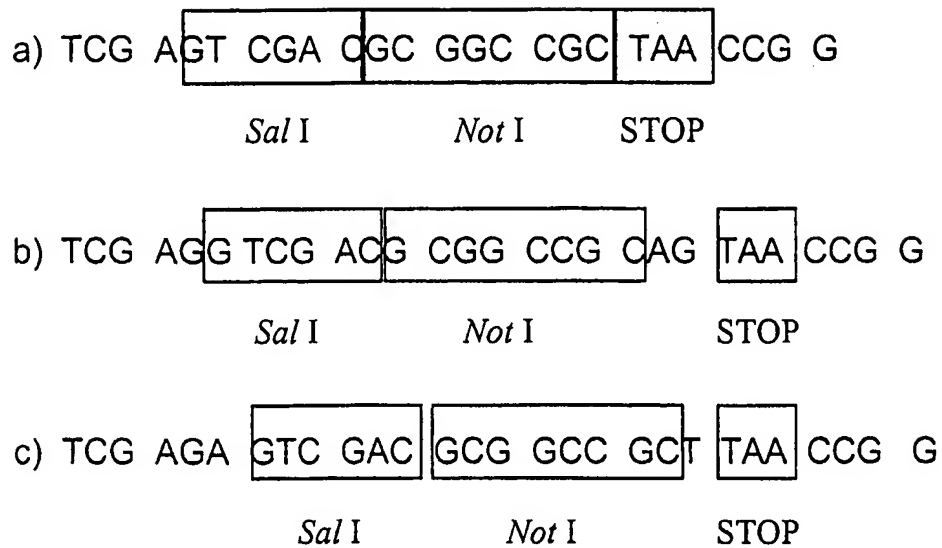
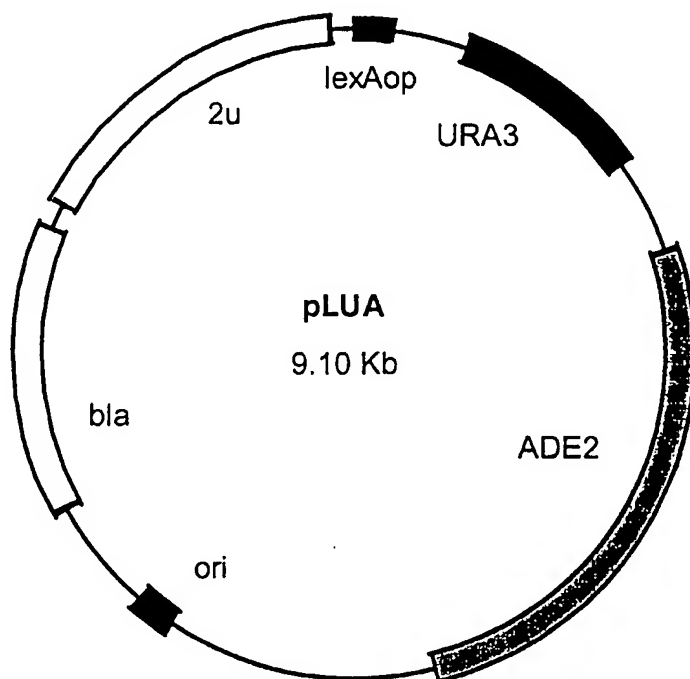


Figure 4 (II) ↑  
Figure 5 ↓



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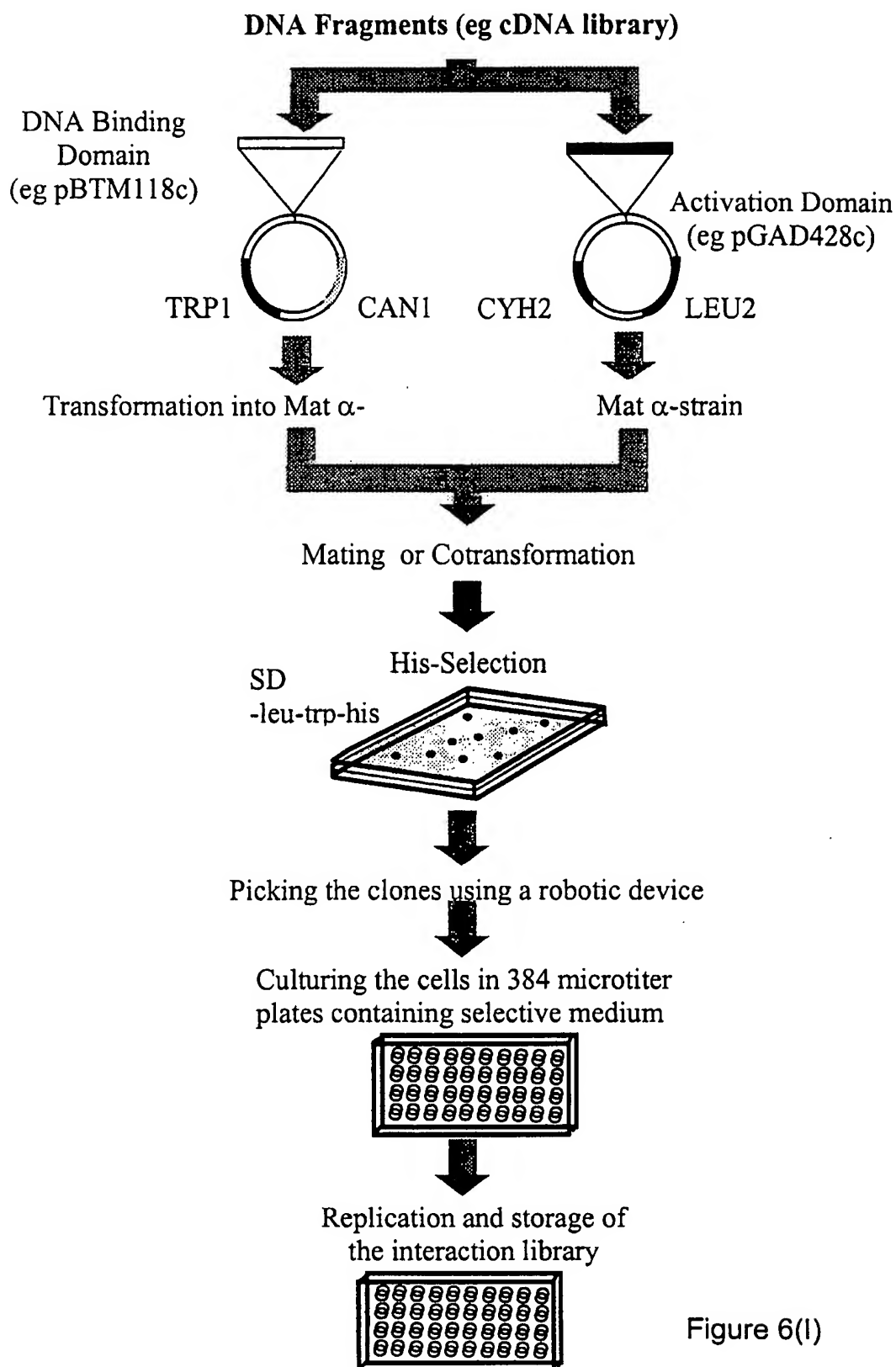


Figure 6(I)

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(continuation from figure 6 (I) )

Spotting of yeast cells onto a nylon membrane;  
Transfer of these membranes to different selective media

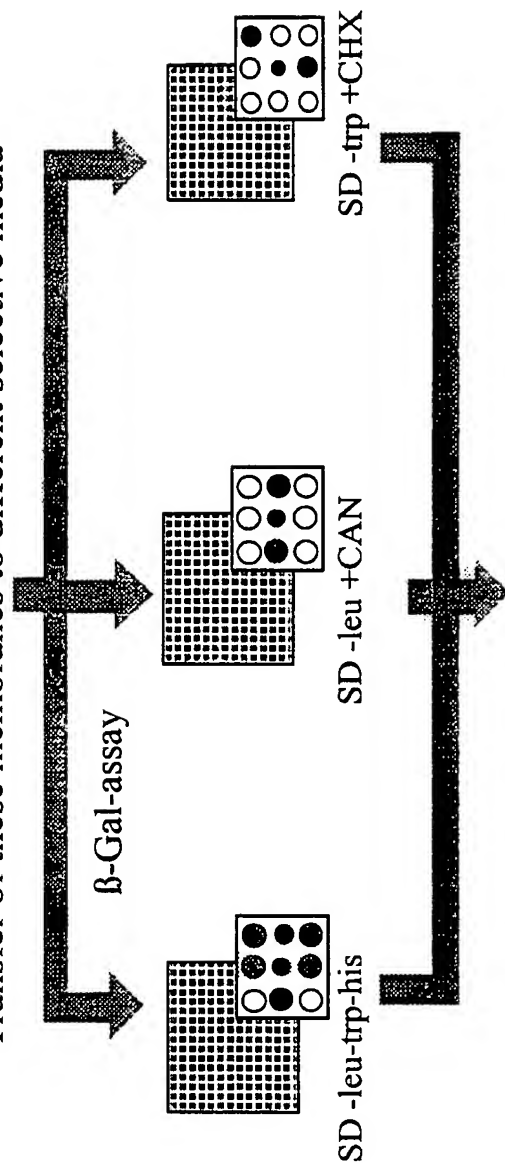
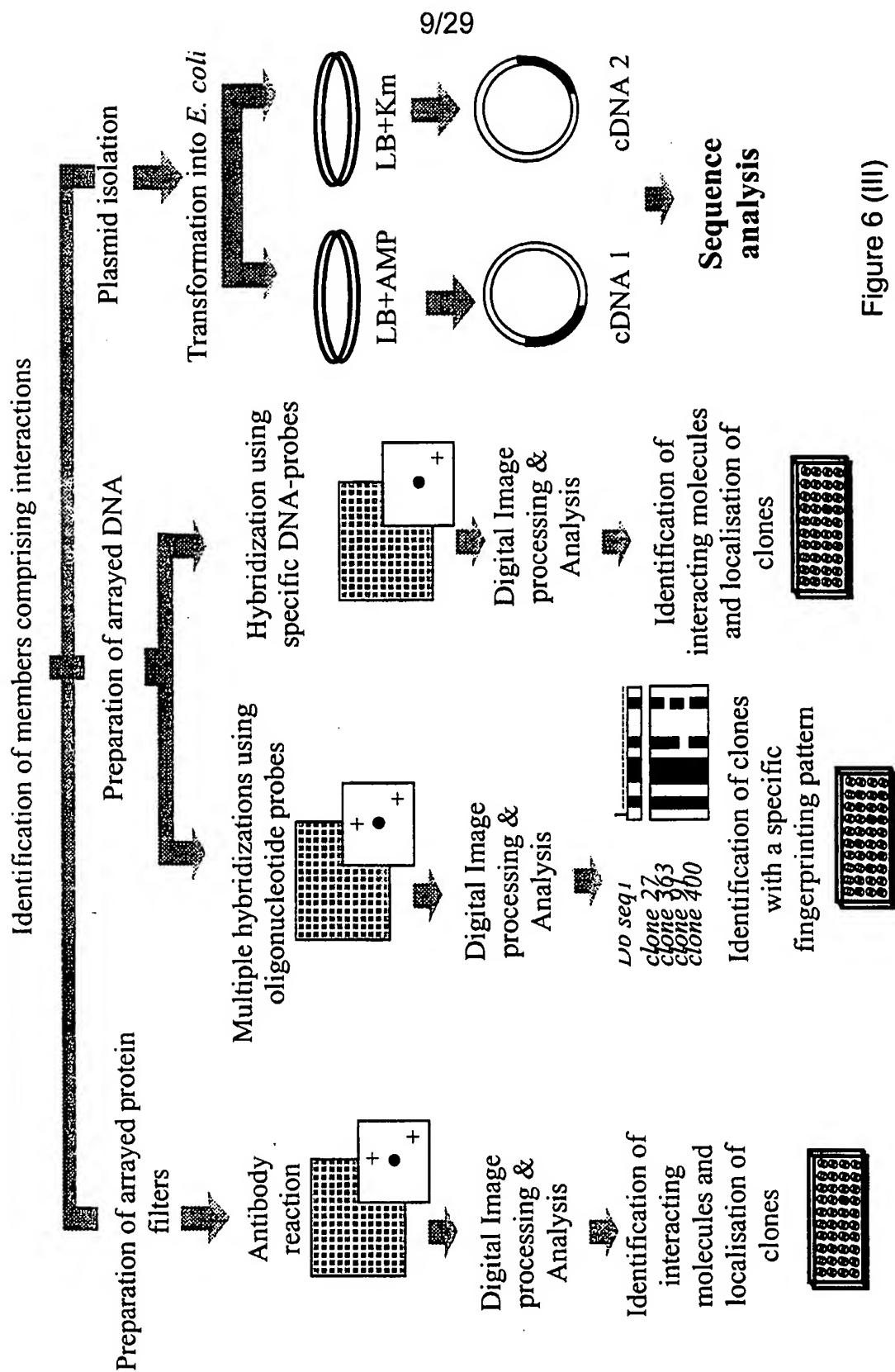
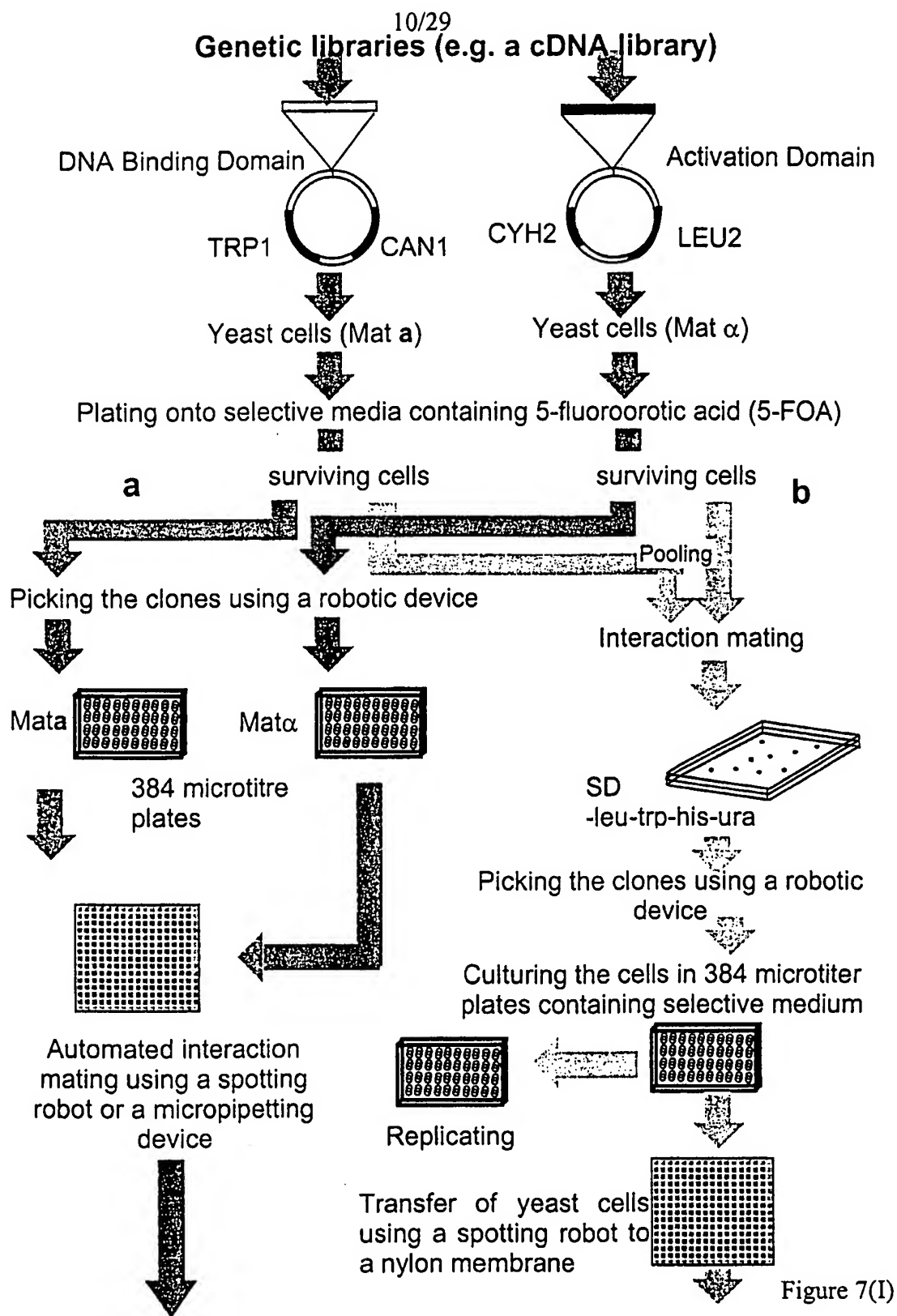


Figure 6 (II)

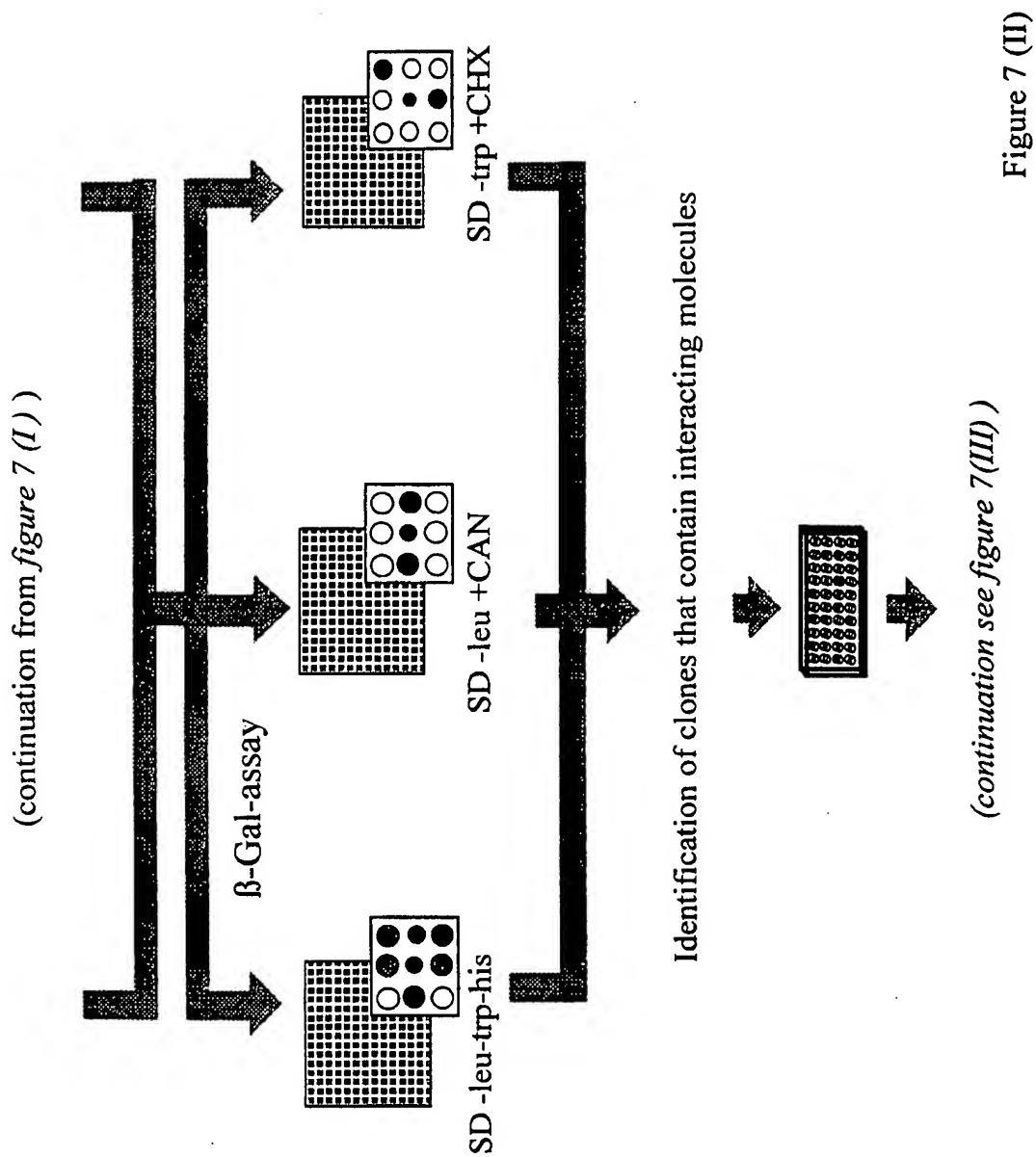
(continuation see figure 6(III) )







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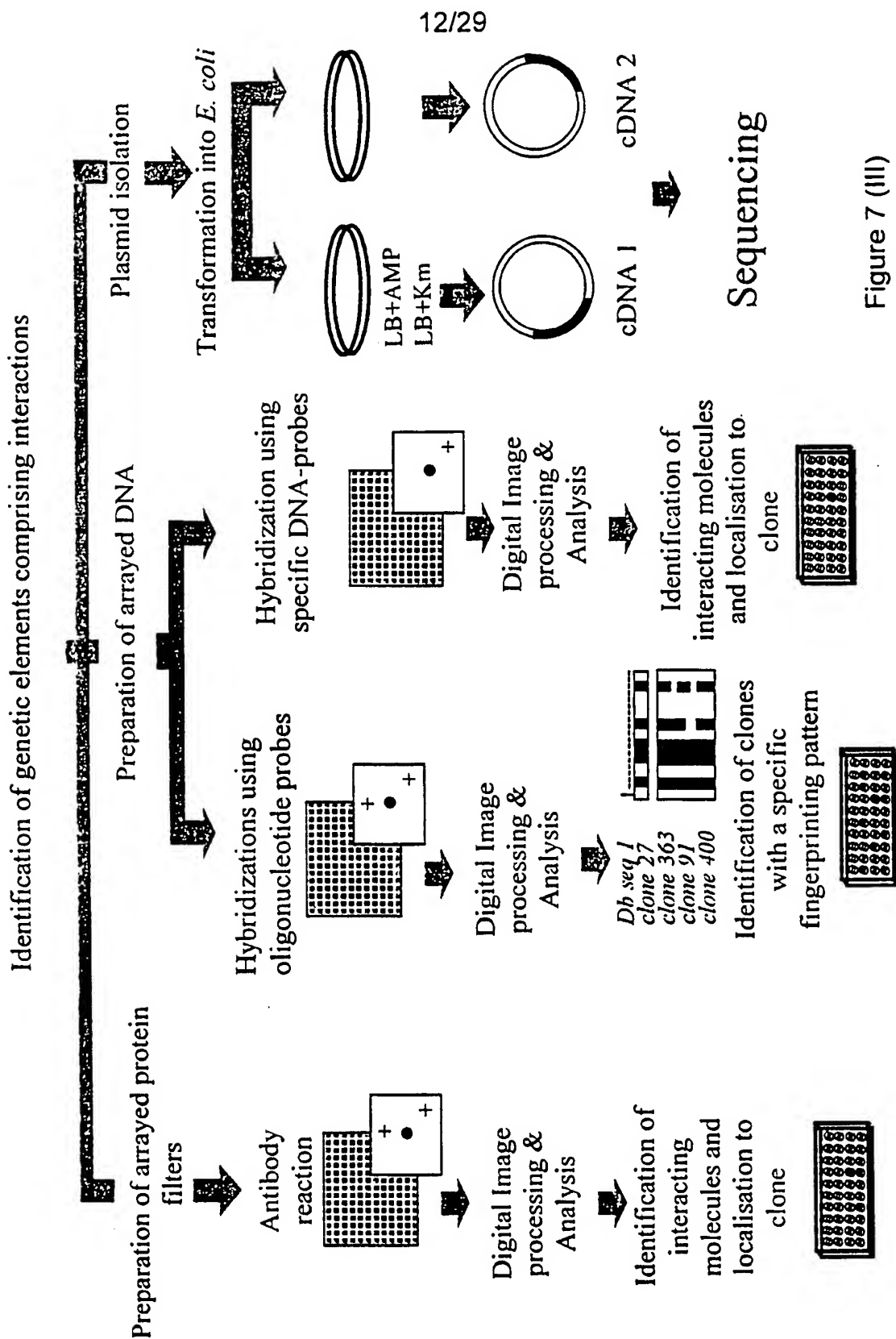


Figure 7 (III)

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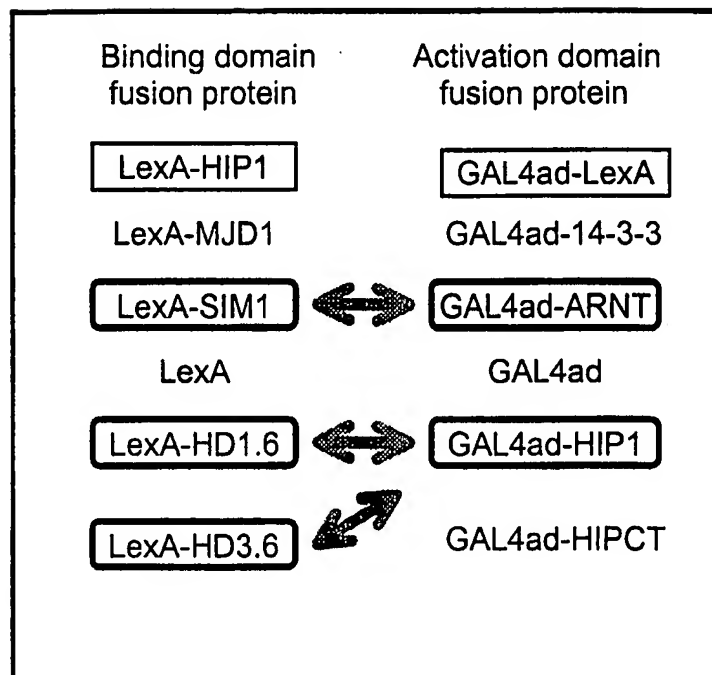


Figure 8

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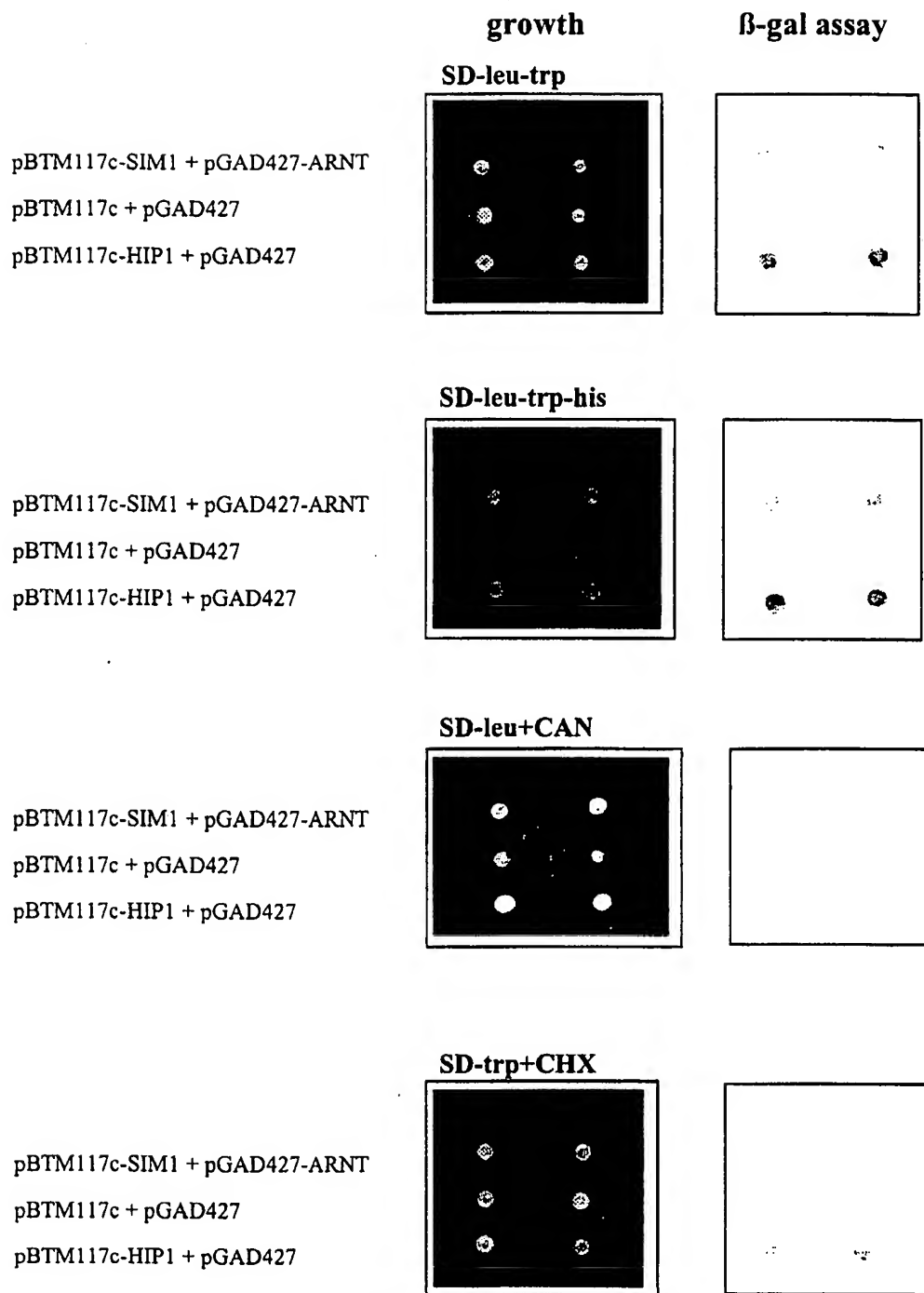


Figure 9

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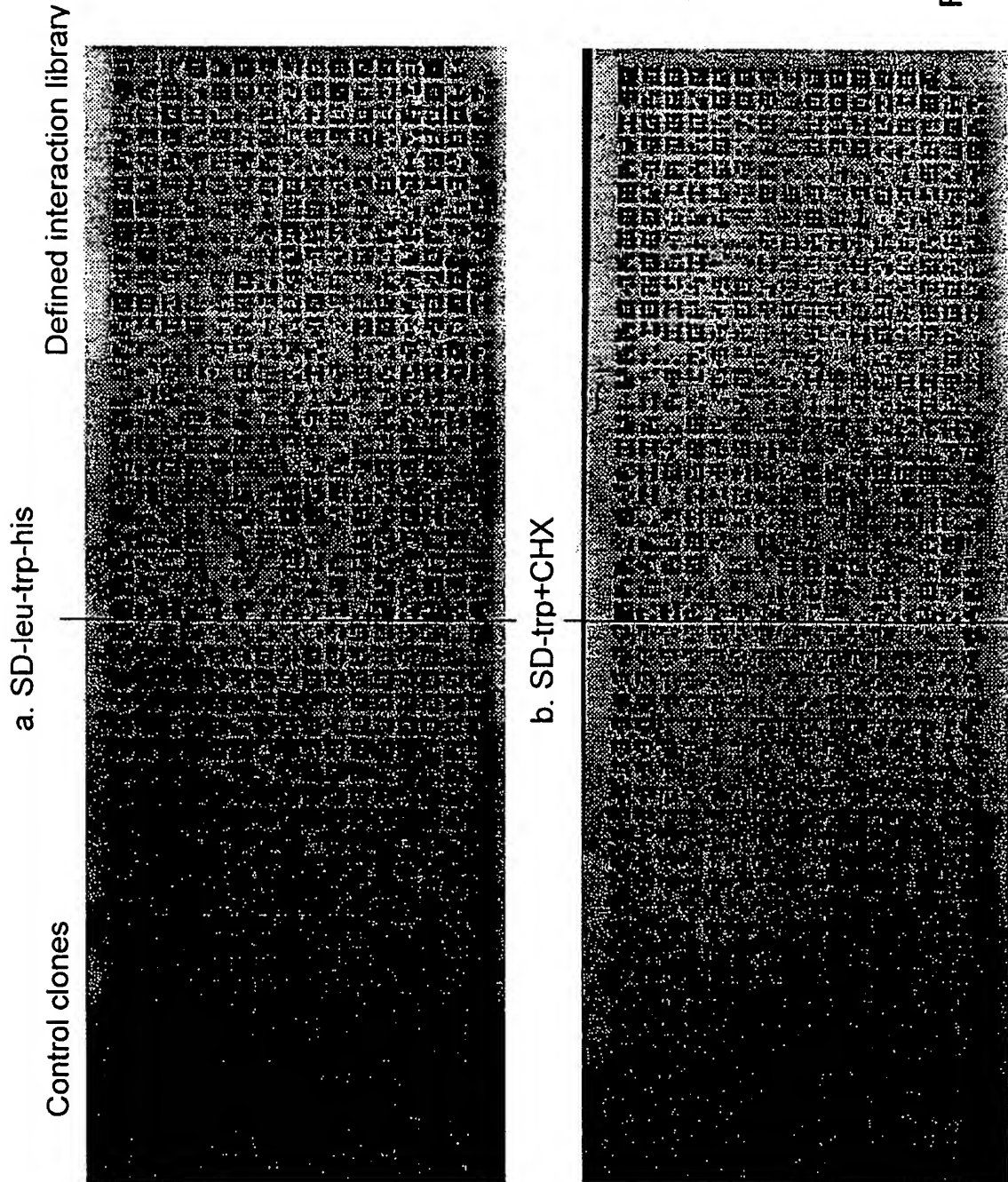


Figure 10 (I)

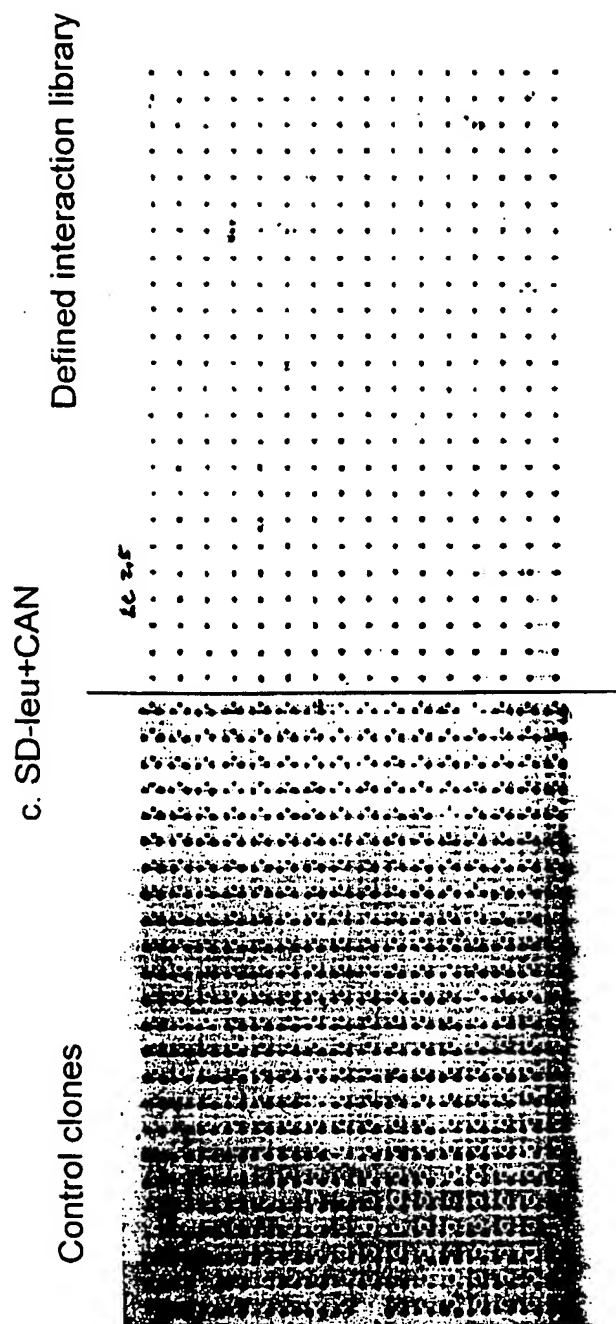


Figure 10 (II)

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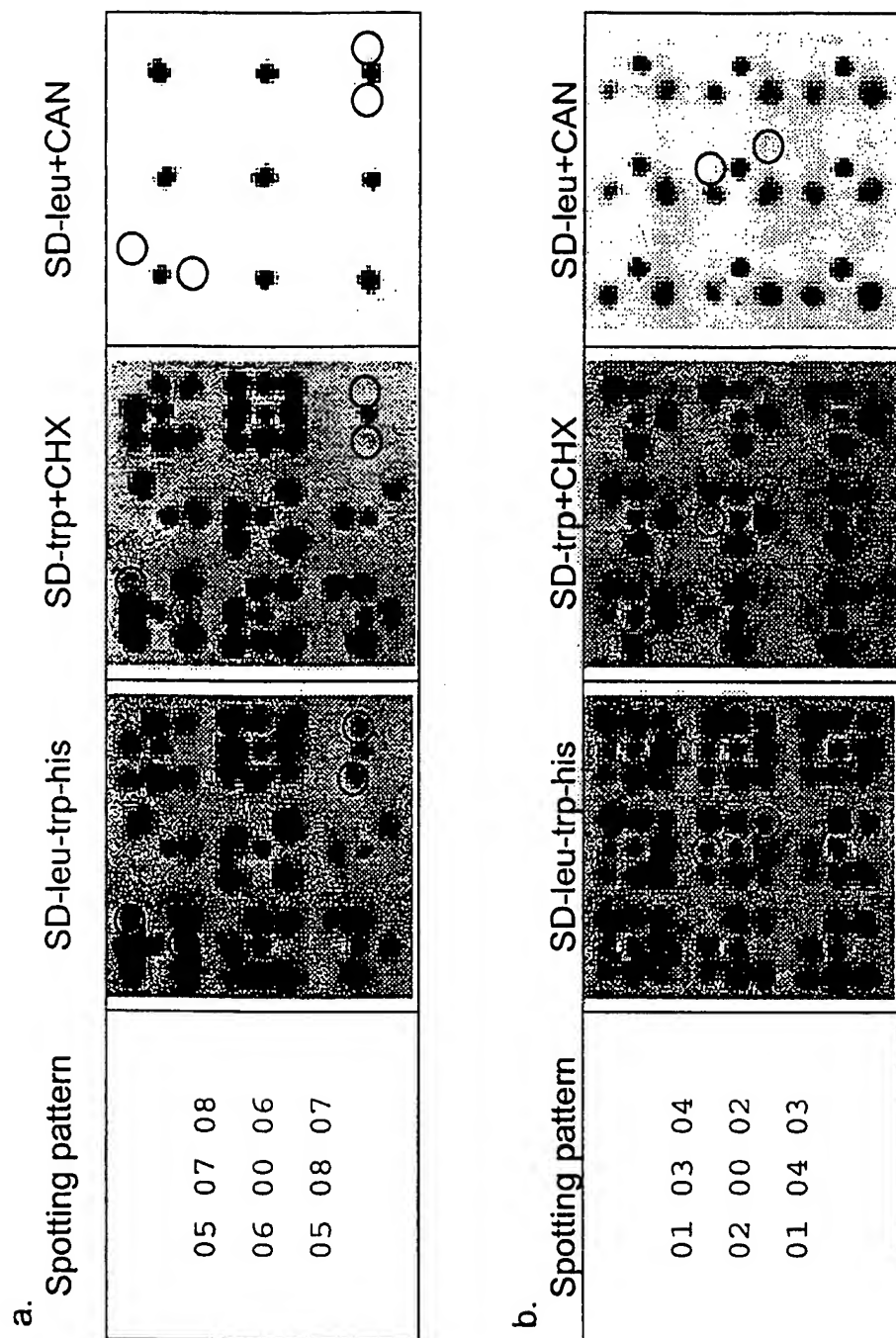


Figure 11

a.

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GPC - PositiveFind				
Clone	SD-leu-trp-his	SD-trp+CHX	SD-leu+CAN	
675 208	2	0	0	
687 2022	2	0	0	
689 2P1	3	0	0	
690 2P2	2	0	0	
693 2P5	3	0	0	
696 2P8	3	0	0	
699 2P12	3	0	0	
709 2P23	2	0	0	
710 2P24	2	0	0	
711 3A2	3	0	0	
712 3A3	3	0	0	
713 3A4	3	0	0	
714 3A5	3	0	0	
716 3A7	3	0	0	
717 3A8	3	0	0	
718 3A11	3	0	0	
719 3A12	2	0	0	
720 3A14	3	0	0	
721 3A15	3	0	0	
722 3A18	2	0	0	
723 3A21	2	0	0	
724 3A23	2	0	0	
725 3A24	3	0	0	
727 3B2	3	0	0	
728 3B3	3	0	0	
729 3B4	3	0	0	
730 3B5	3	0	0	
731 3B6	3	0	0	
732 3B7	3	0	0	
733 3B8	3	0	0	
734 3B9	3	0	0	
735 3B10	3	0	0	
736 3B12	3	0	0	
737 3B13	3	0	0	
738 3B14	3	0	0	
739 3B15	3	0	0	
741 3B17	2	0	0	

b.

GPC - PositiveFind				
Clone	SD-leu-trp-his	SD-trp+CHX	SD-leu+CAN	
1733 6L22	3	0	0	
2302 8N24	2	0	0	

Figure 12



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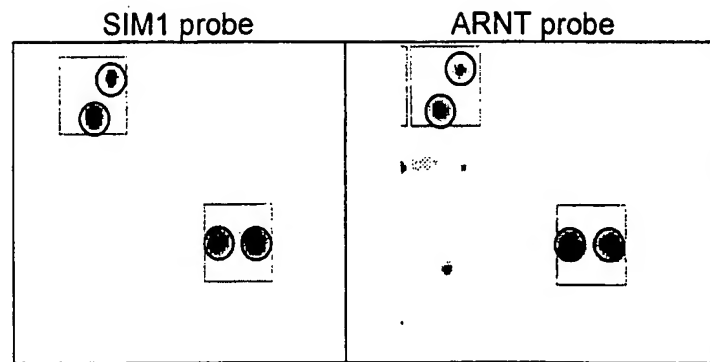


Figure 13

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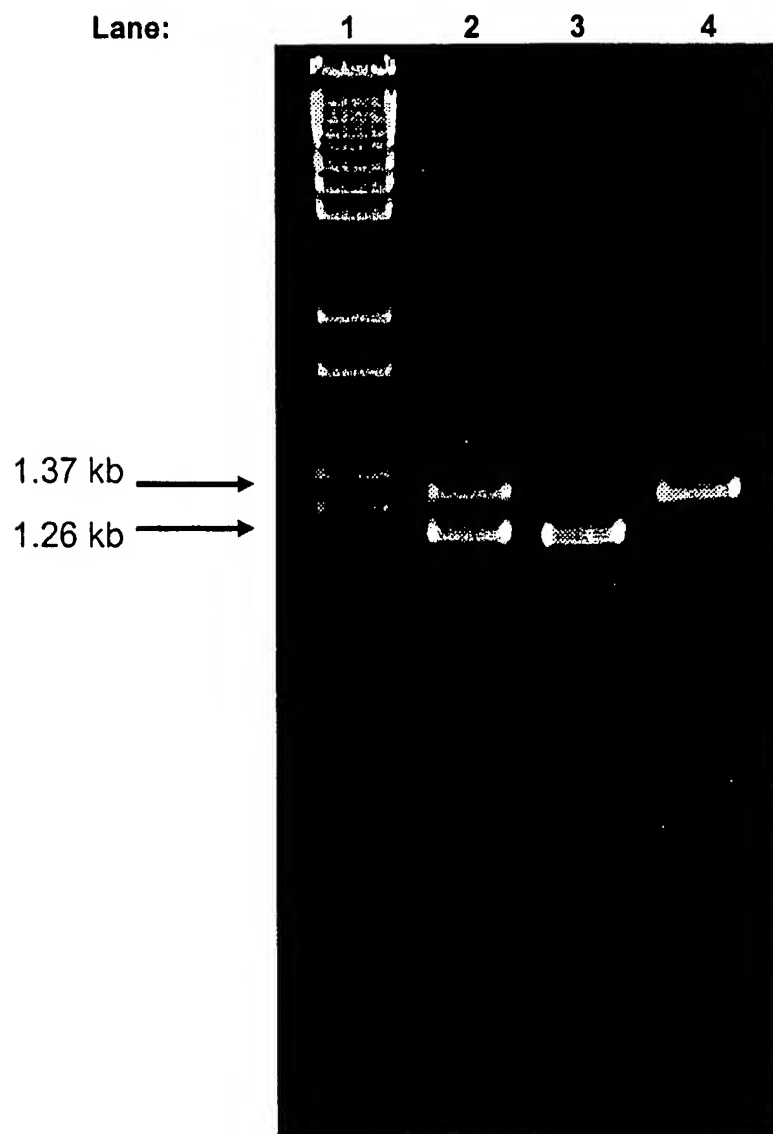


Figure 14

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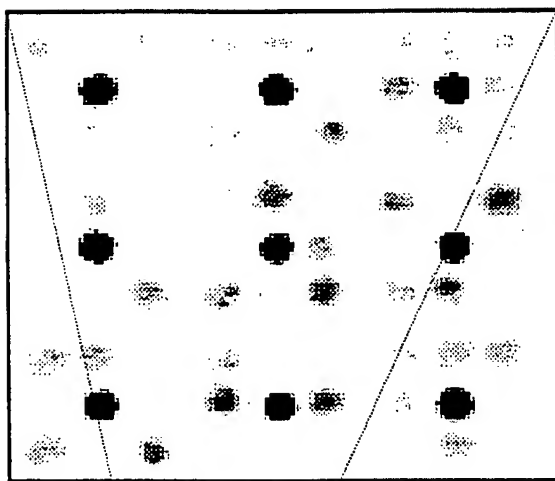
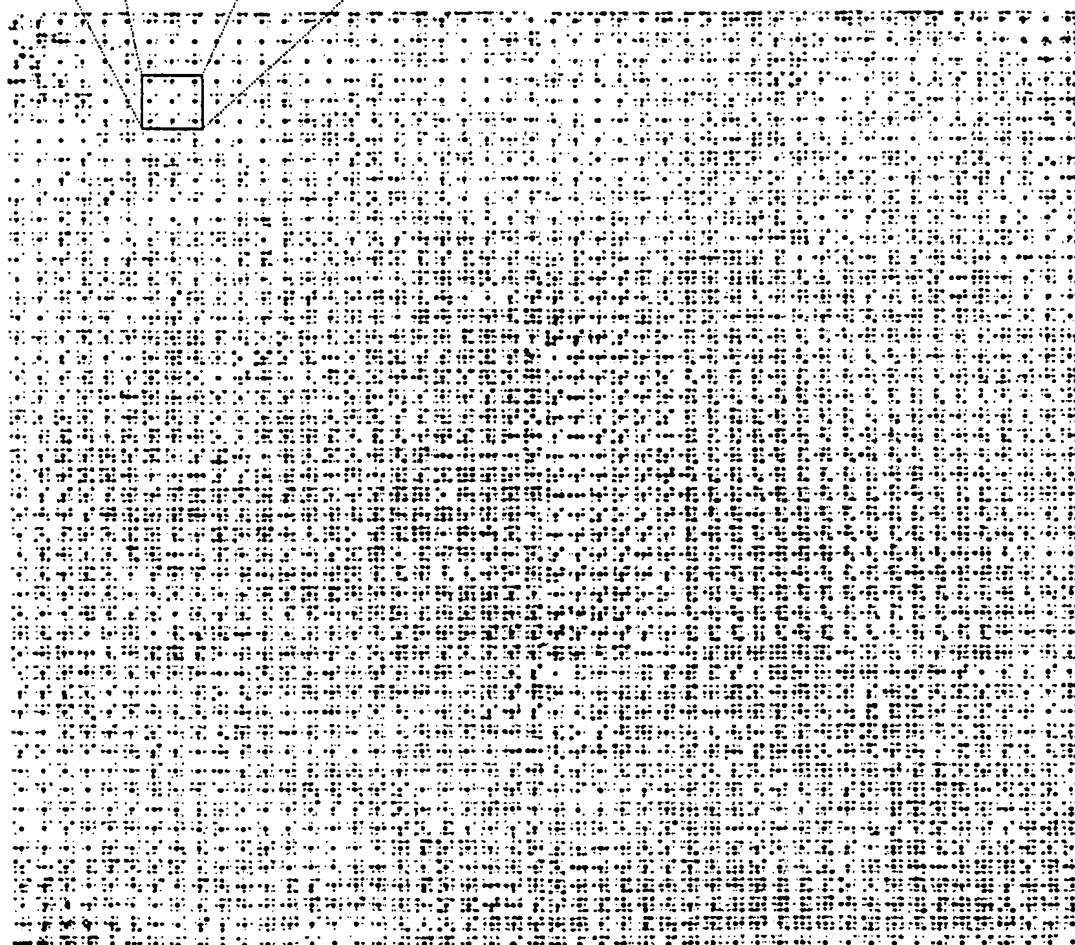
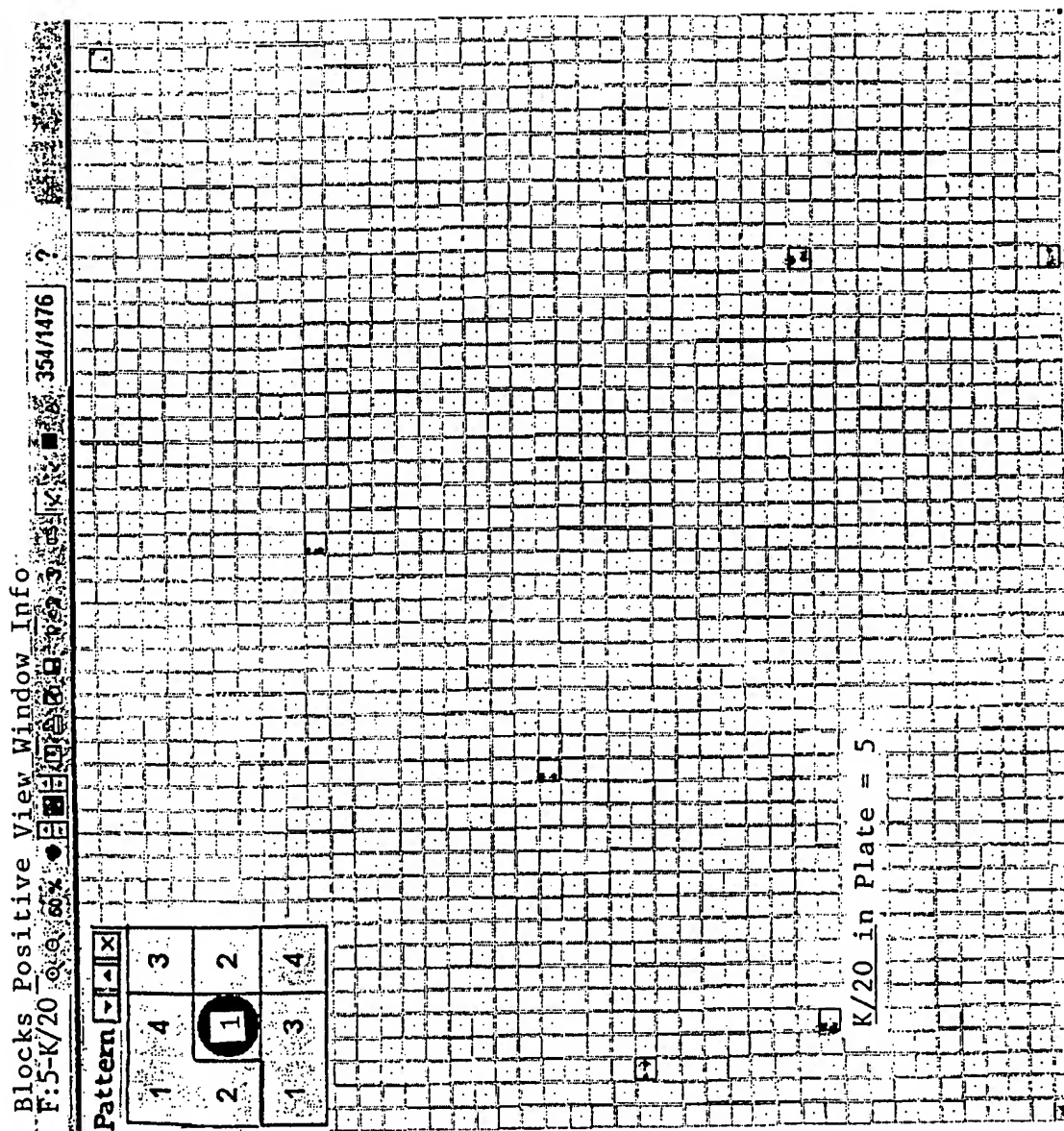


Figure 15



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Figure 16



1st Hybridisation

Protein A: ●

Interaction Map

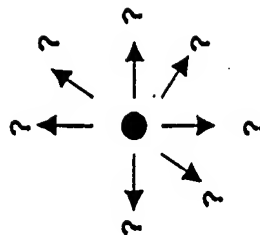
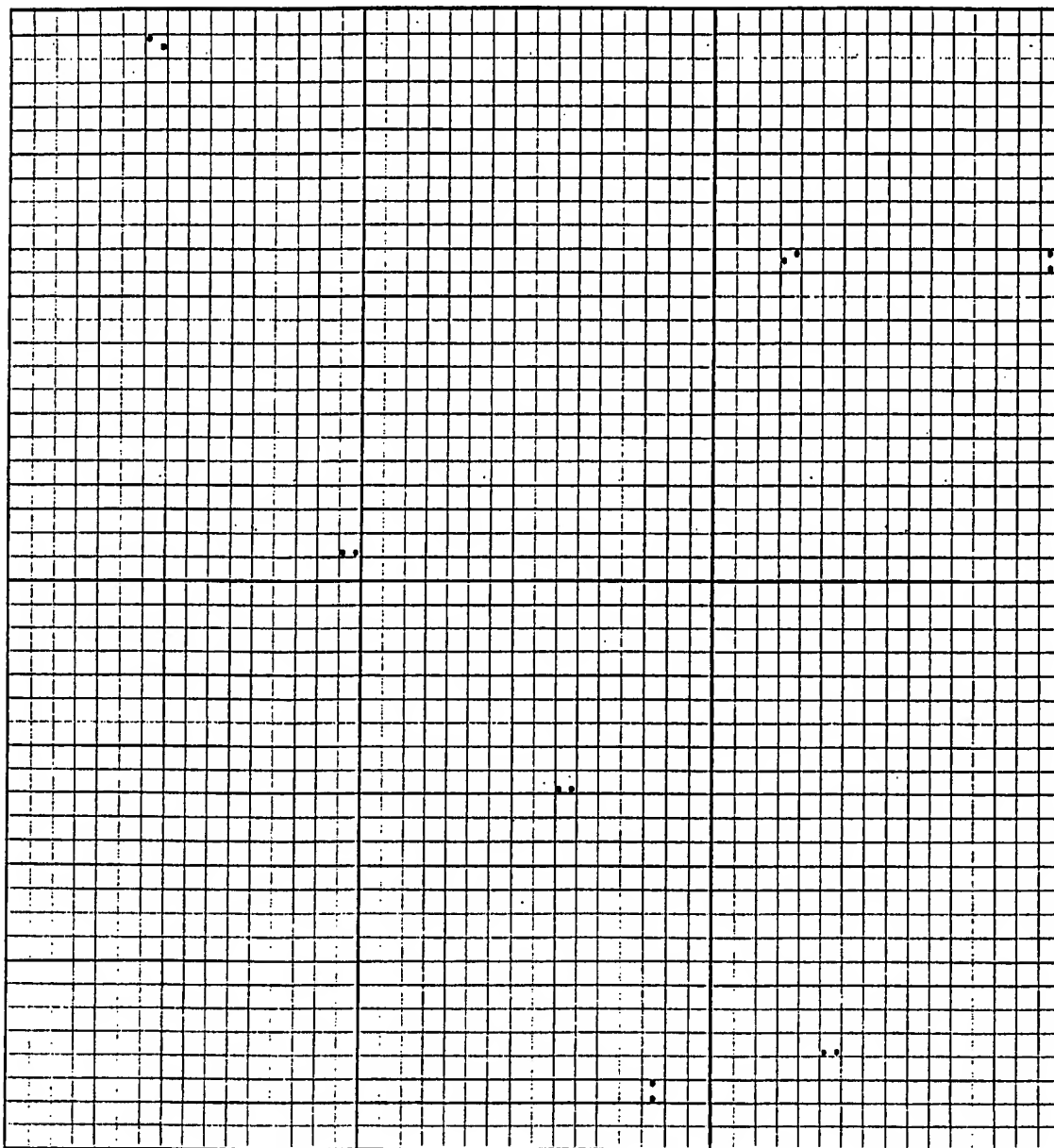
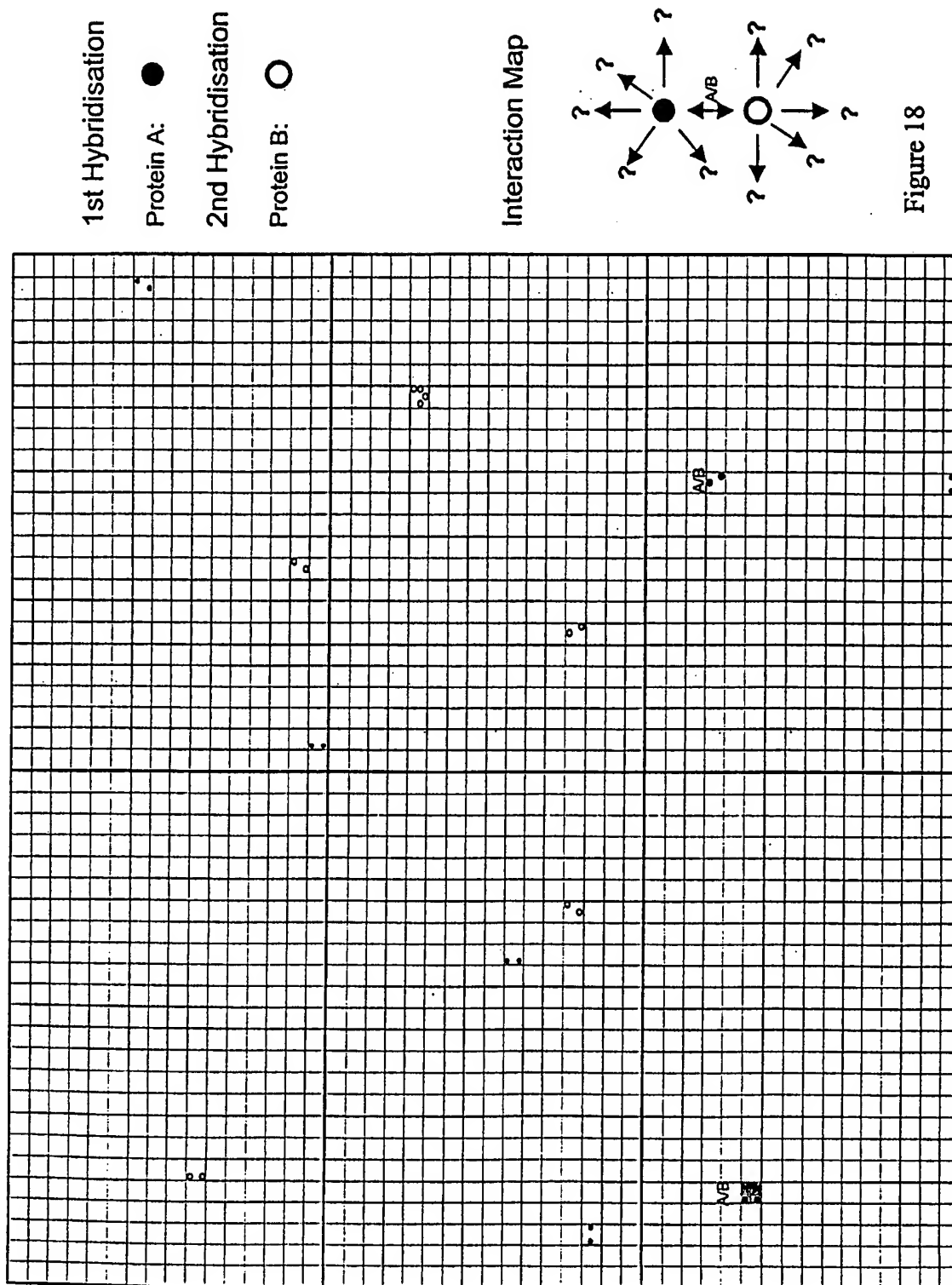
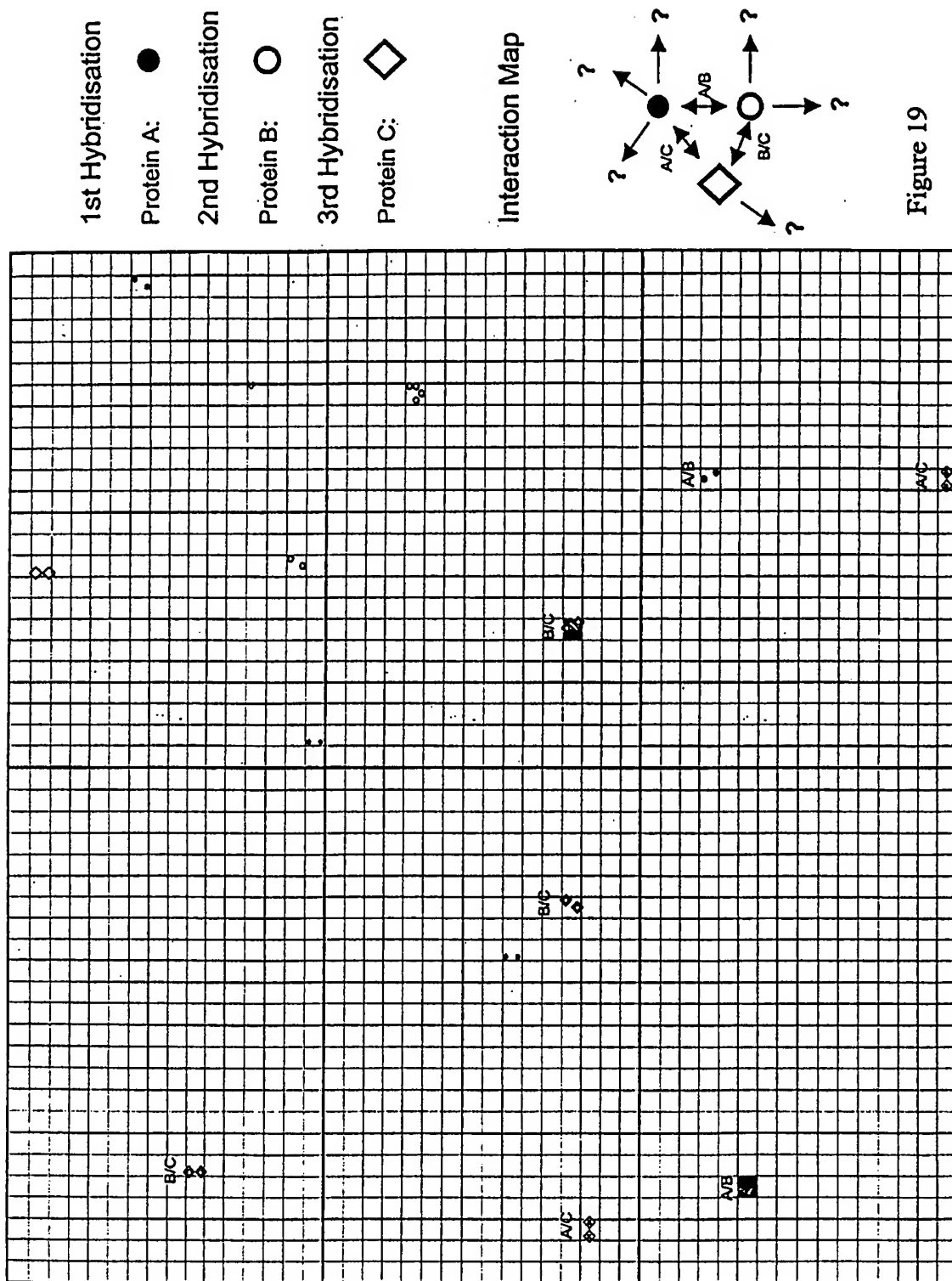


Figure 17







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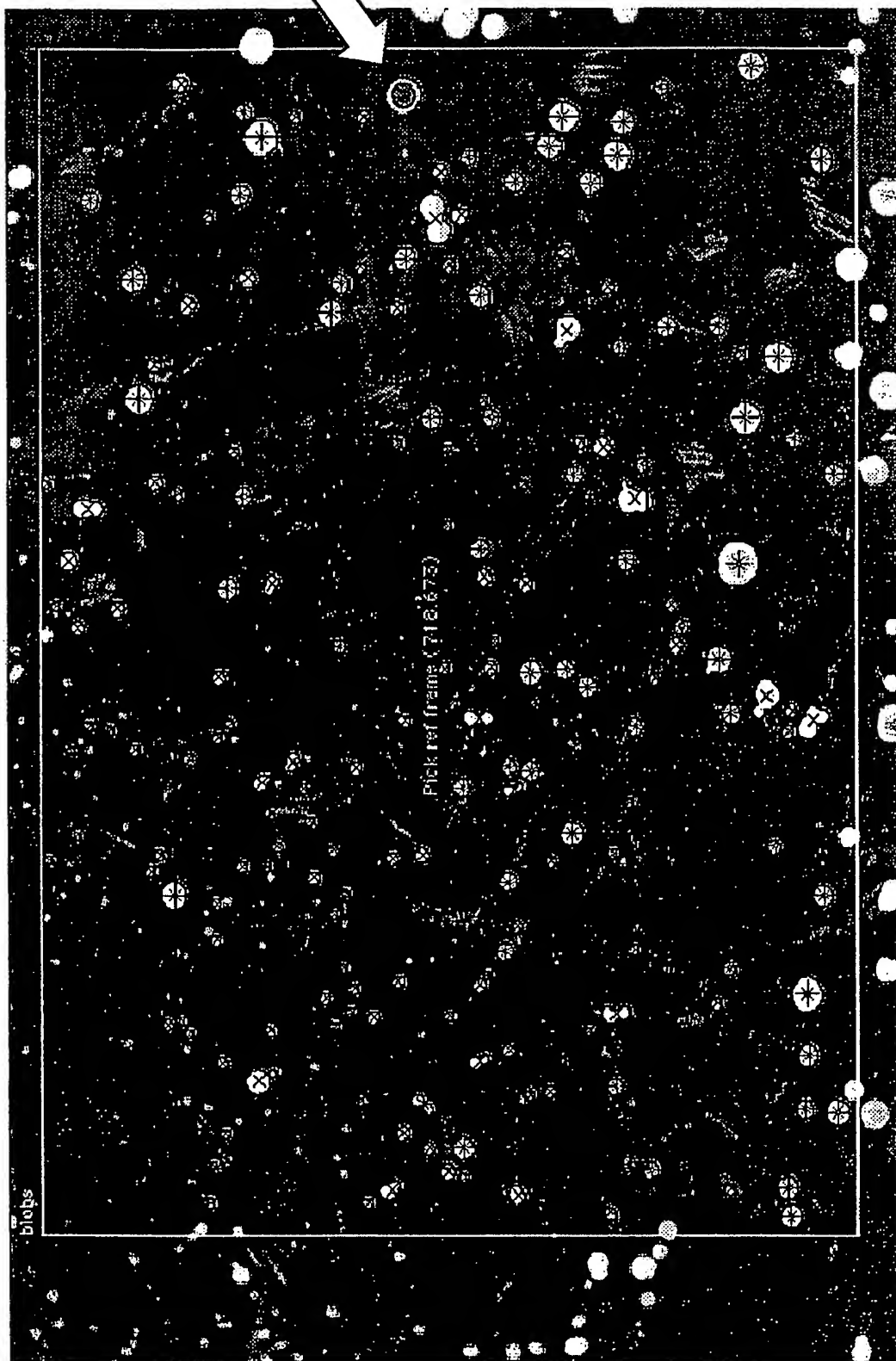


Figure 20



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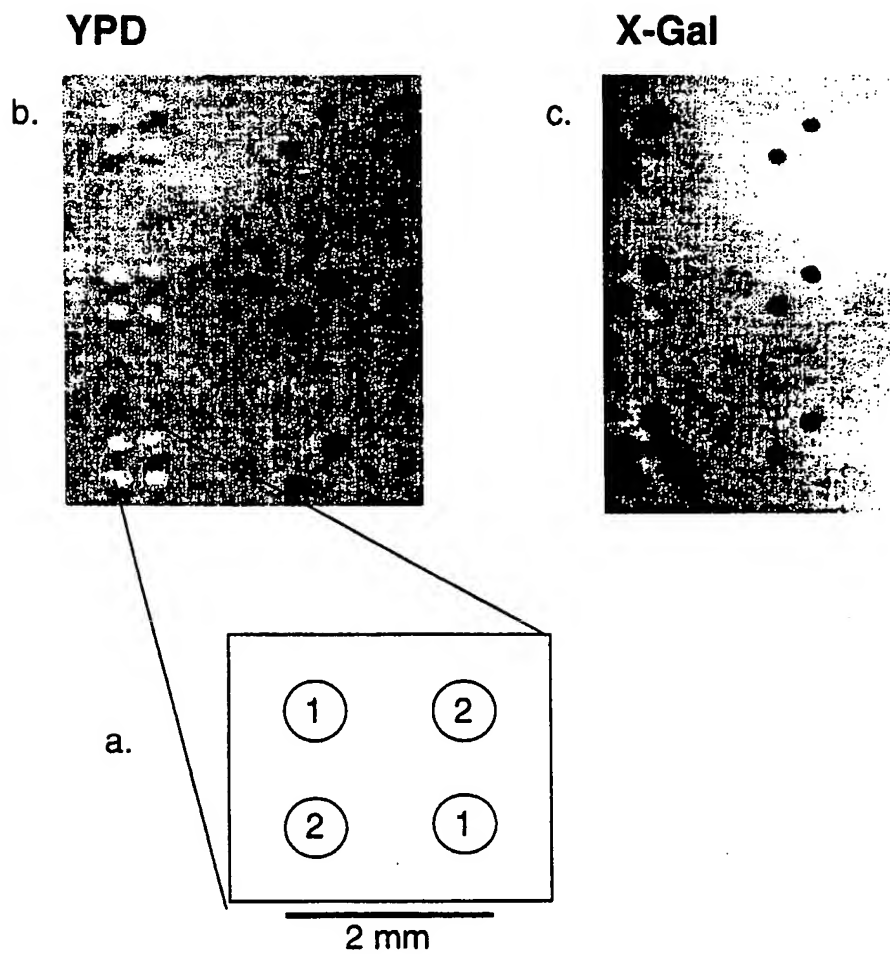


Figure 21

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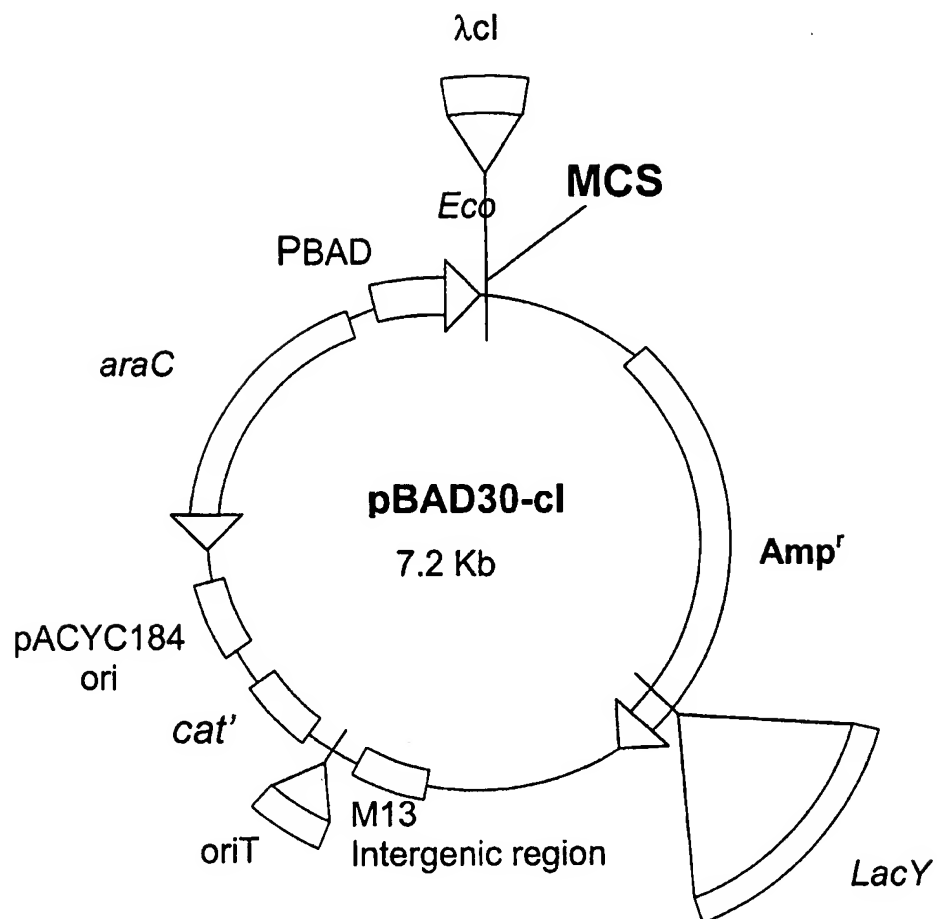


Figure 22 (I)

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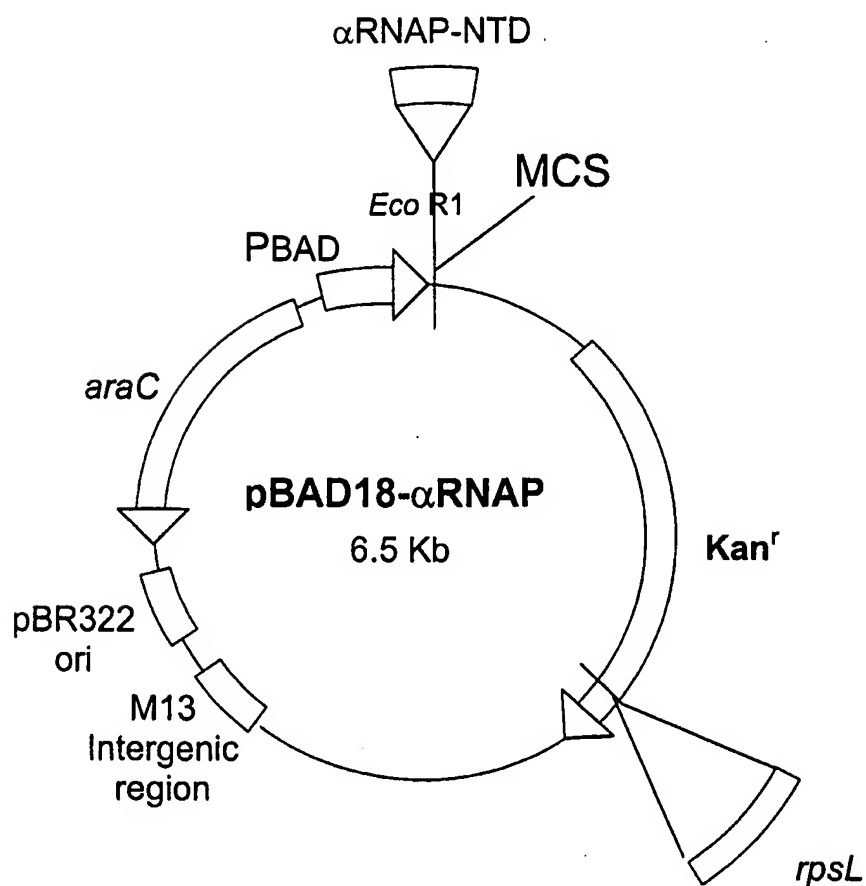


Figure 22 (II)

# INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 98/07656

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 G01N33/50 G01N33/68

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 G01N C12N C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 96 32503 A (GEN HOSPITAL CORP ;UNIV JOHNS HOPKINS (US)) 17 October 1996 see page 3, line 10 - page 4, line 9 ---	1-32, 34-36
A	EP 0 790 304 A (AMERSHAM INT PLC) 20 August 1997 see column 11 - column 12 ---	1-32, 34-36
A	DOVE S L ET AL: "ACTIVATION OF PROKARYOTIC TRANSCRIPTION THROUGH ARBITRARY PROTEIN-PROTEIN CONTACTS" NATURE, vol. 386, 10 April 1997, pages 627-630, XP002050708 see the whole document --- -/--	1-32, 34-36

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

\* Special categories of cited documents:

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"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

22 April 1999

Date of mailing of the international search report

28/04/1999

Name and mailing address of the ISA

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Fax: (+31-70) 340-3016

Authorized officer

Hoekstra, S

# INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 98/07656

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A,P	WO 97 47763 A (CURAGEN CORP) 18 December 1997 see the whole document ---	1-32, 34-36
A,P	WO 98 07845 A (JOUNG J KEITH ;DOVE SIMON (US); HARVARD COLLEGE (US); HOCHSCHILD A) 26 February 1998 see claims ---	1-32, 34-36
A	BARTEL, P. ET AL.: "Elimination of false positives that arise in using the two-hybrid system" BIOTECHNIQUES, vol. 14, no. 6, 1993, pages 920-924, XP002004036 see the whole document -----	1-32, 34-36

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/EP 98/ 07656

## Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 31-33, 37-61  
because they relate to subject matter not required to be searched by this Authority, namely:  
Claims 37-61 have been excluded from the search in view of: Rule 39.1(vi) PCT relating to program for computers; Rule 39.1(v) PCT relating to presentation of information.
2. ☒ Claims Nos.: 31-33  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:  
see FURTHER INFORMATION sheet PCT/ISA/210
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this International application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Claims Nos.: 31-33

Claims 31-33 are methods for preparing compositions which are technically limited to molecules identified by the screening methods of claims 1-30. No features of a technical nature are comprised in the claims to either characterise the method steps or these molecules. These claims do hence not comply with all the prescribed requirement of Article 6 and Rule 6.3(a) PCT to such an extend that a meaningful search is not possible for these claims.

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/EP 98/07656

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9632503 A	17-10-1996	AU 5541296 A CA 2217545 A EP 0830459 A JP 11502717 T	30-10-1996 17-10-1996 25-03-1998 09-03-1999
EP 0790304 A	20-08-1997	AU 1165697 A EP 0868509 A WO 9723609 A	17-07-1997 07-10-1998 03-07-1997
WO 9747763 A	18-12-1997	AU 3395597 A CA 2257958 A	07-01-1998 18-12-1997
WO 9807845 A	26-02-1998	AU 4159697 A	06-03-1998